THE CHARACTERISATION OF A FENOPROFEN PRODRUG

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I do not know what I may appear to the world, but to myself I seem to have been only like a boy playing on the seashore, and diverting myself in now and then finding a prettier shell, or a smoother pebble than ordinary, whilst the great ocean of truth lay all undiscovered before me. – Isaac Newton

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Chapter 1: The prodrug concept

1.1 Introduction

The term "prodrug" refers to a pharmacologically inactive compound that is converted to an active drug by a metabolic biotransformation (Silverman, 1992: 352). A nonenzymatic process, such as hydrolysis, can activate a prodrug *via* several routes (Bundgaard & Friis, 1996: 352).

Prodrugs are mostly used to improve a drug's physical and chemical properties in order to optimise drug delivery. Not only can prodrugs prolong therapeutic effect, but side-effects can also be minimised.

1.2 General applications of prodrugs

In general prodrugs are used to overcome a drug's major disadvantages (illustrated in figure 1.1):

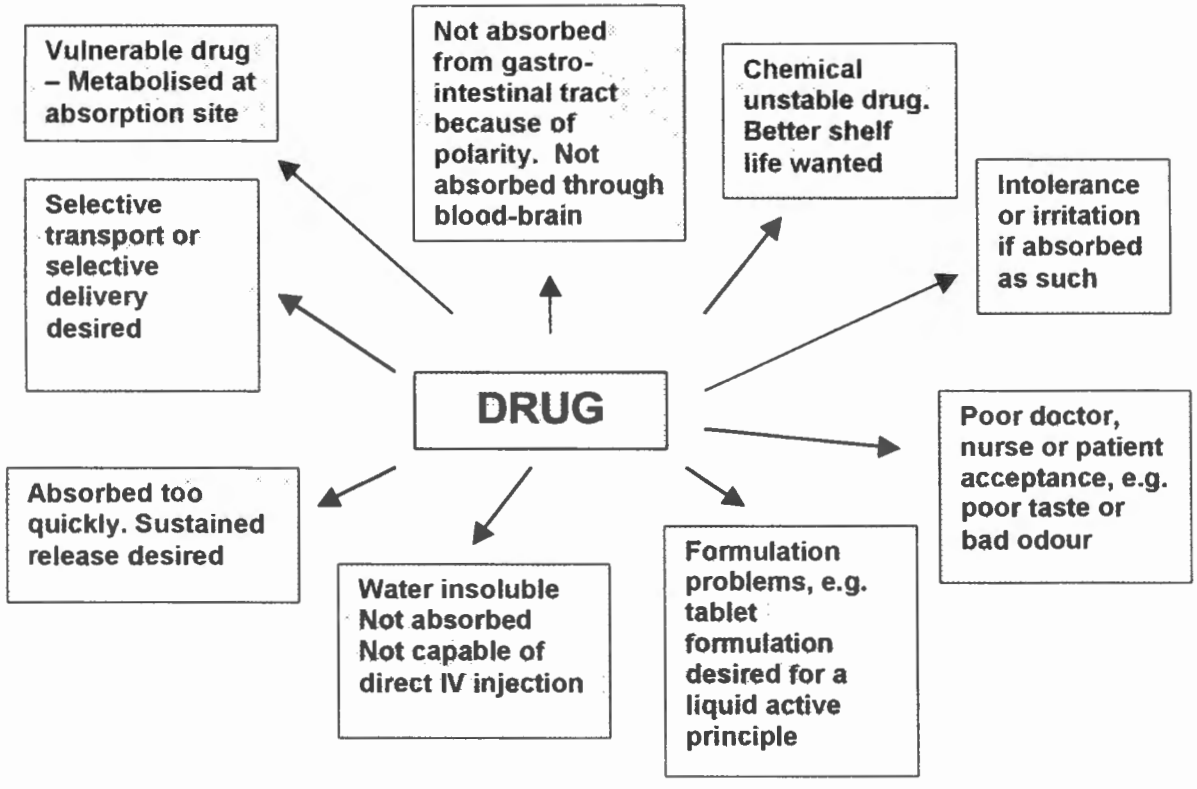


Figure 1.1: Shortcomings that may be overcome by using prodrugs (Wermuth *et al.*, 1996: 674).

In the manufacturing practice, prodrugs are usually utilised to:

- Increase lipophilicity
- Increase duration of pharmacological effects

- Increase site specificity
- Decrease toxicity and adverse effects
- Improvement in drug formulation, like stability, water solubility or suppression of an unwanted physicochemical property (Wermuth *et al.*, 1996: 674, Nogrady, 1985: 388, (Bundgaard & Friis, 1996: 353 and Fleming, 1995: 172).

1.3 Types of prodrugs

There are several classifications of prodrugs. Some drugs were not originally designed as prodrugs, but it was later discovered that they undergo biotransformation in the body and are actually prodrugs. Most of the time a new prodrug can be designed, because its metabolic pathway can be predicted. This way of "designing a prodrug" is termed "drug latentiation" (Silverman, 1992: 354). The term "drug latentiation" has been refined into two classes that are called bioprecursors and carrier-linked prodrugs (Wermuth *et al.*, 1996: 672 and Fleming, 1995: 172).

1.3.1 Bioprecursors

A bioprecursor is a compound that is metabolised by molecular modification into a new compound, that forms the active drug (Wermuth, *et al.*, 1996: 697). This type of prodrug differs from a carrier-linked prodrug in the sense that a carrier-linked prodrug is an active drug linked to a carrier that generally is released by a hydrolytic reaction. A bioprecursor contains a different structure that cannot be converted to the active drug by simple cleavage of a group from the prodrug, they are converted to active metabolites *in vivo*, which then in turn exhibit the pharmacological action (Wermuth *et al.*, 1996: 672). Examples of such metabolites are given in table 1.1.

Table 1.1: Bioprecursors and their active metabolites (Wermuth, *et al.*, 1996: 698).

Bioprecursor	Active metabolite
Acetanilide	Paracetamol
Proguanil	Cycloguanil
Imipramine	Desmethylimipramine
Chloral hydrate	Trichloroethanol
Phenylbutazone	Oxyphenylbutazone
L-dopa	Dopamine

For the purpose of this study only carrier-linked prodrugs will be discussed further because most of the nonsteroidal anti-inflammatory drugs (NSAIDs), contain carboxylic functional groups, which offer a number of possibilities for carrier-linked derivative formation.

1.3.2 Carrier-linked prodrugs

This type of prodrug (illustrated in figure 1.2) consists of a carrier group linked to the active drug molecule, for example an ester group that will be hydrolysed to give an active carboxylic acid, being the drug.

Once in the body enzymatic attack to the prodrug molecule releases the active drug. These prodrugs can thus be regarded as drugs containing specialised non-toxic protective groups to alter or eliminate undesirable properties in the parent molecule (Wermuth, *et al.*, 1996: 672).

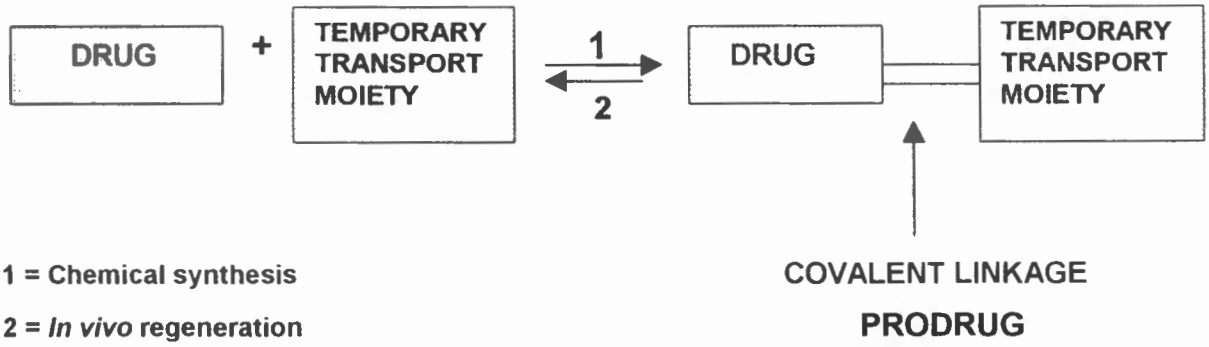


Figure 1.2: The carrier–linked prodrug principle (Wermuth, *et al.*, 1996: 673).

According to Wermuth *et al.*, (1996: 673), a well-designed carrier-linked prodrug must comply with the following criteria:

- The linkage between the drug substance and the transport moiety is usually a covalent bond.
- The prodrug must be inactive or less active than the parent compound.
- The linkage between the parent compound and the transport moiety must be broken *in vivo.*
- The prodrug as well as the transport moiety (which will be released later) must be non-toxic.
- The liberation of the active form *in vivo* must be rapid so that an effective drug level in the blood can be reached.
- Rapid metabolisation or drug inactivation must be minimised at the site of action.

Carrier-linked prodrugs can be subdivided further into bipartate, tripartate and mutual prodrugs (Silverman, 1992: 354). A bipartate prodrug is a prodrug comprised of one carrier attached to the drug, as illustrated in figure 1.3.

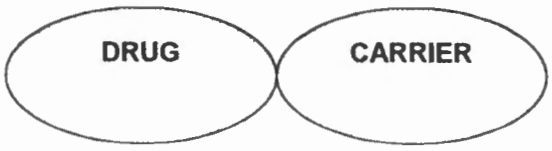


Figure 1.3: The bipartate prodrug principle.

A tripartate prodrug consists of a carrier connected to a linker arm that is connected to the drug as illustrated in figure 1.4.

Figure 1.4: The tripartate prodrug principle.

A mutual prodrug consists of two, usually synergistic drugs attached to each other (one drug forms the carrier for the other and *vice versa*) (Silverman, 1992: 354), as illustrated in figure 1.5.

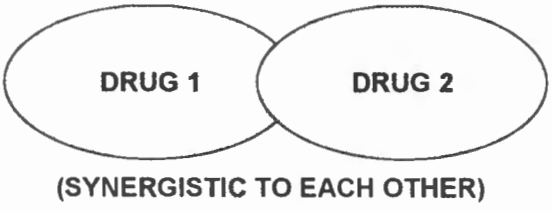


Figure 1.5: The mutual prodrug principle.

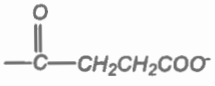
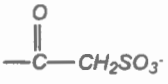
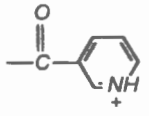
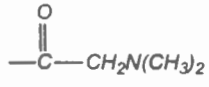
1.3.2.1 Mechanisms of carrier-linked prodrug activation

The most common reaction for activation of carrier-linked prodrugs is hydrolysis. The general functional groups involved are (Silverman, 1992: 355):

- Alcohols and carboxylic acids
- Amines
- Carbonyl compounds

Only alcohols and carboxylic acids will be discussed further, because these groups are the most commonly used, not only in the creation of carrier-linked NSAID prodrugs but also in general prodrug synthesis, because esterase enzymes are so abundant in the human body. It is also possible to prepare ester derivatives with virtually any degree of hydrophilicity or lipophilicity (Silverman, 1992: 356). Ester analogs of alcohols that can be used to create prodrugs are illustrated in table 1.2.

Table 1.2: Ester analogs of alcohols as prodrugs (Silverman, 1992: 356).

Drug-OH ⇨ Drug-OR	
R	**Effect**
	R = Aliphatic or aromatic groups Decreases water solubility.
	Increases pK_a (~5)
	Increases pK_a (~1)
	Increases pK_a (~4)
	Increases pK_a (~8)

Esters are also very stable molecules and a multitude of ester prodrugs can therefore be prepared to solve a wide variety of problems that require a prodrug approach (Wermuth, *et al.*, 1996: 676) as is illustrated in table 1.3:

Table 1.3: Functional groups to be added to alcohol or carboxylic acid containing drugs (Silverman, 1992: 357).

Drug functional group	Added functional group	Result
Alcohol containing drugs	• Aliphatic or aromatic carboxylic acids. • Carboxylic acids containing amino or additional carboxylate groups. or • Phosphate or sulphate esters.	• Water solubility decreases. • Water solubility increases.
Carboxylic acid containing drugs	• Long chain aliphatic or sterically hindered esters. • Electron withdrawing groups on alcohol groups.	• Rate of hydrolysis decreases. • Rate of hydrolysis increases.

Due to the wide variety of esterases present in the target tissues of oral prodrug regeneration, it is not surprising that esters are the most numerous prodrugs designed so far. Because so many esterase enzymes are present in the liver, blood and gastro-intestinal walls, ester prodrugs are the first choice when a prodrug must be administrated orally (Sinkula & Yalkowsky., 1975: 181, Zorc *et al.*, 1993: 127 and Whitehouse & Rainsford., 1993: 795).

A big problem with the use of this specific prodrug approach is that in some cases the esters are not very good substrates for the endogenous esterases, sulphatases or phoshatases, or may not be hydrolysed at a rapid enough rate (Silverman, 1992: 356). When this problem occurs, a different ester can be used, or an electron withdrawing or donating group can be attached.

1.3.3 Macromolecular prodrugs

Macromolecular prodrugs are a type of carrier-linked prodrug that have drawn a considerable amount of attention the last few years. Macromolecular prodrugs differ from ordinary carrier-linked prodrugs in the sense that the drug carrier is a macromolecule. Proteins, synthetic polymers or polysaccharides (such as dextran) are various types of macromolecules used as drug carriers (Bogdansky, 1990: 239). Two types of macromolecular prodrugs exist. The first type consists of polymers, including nanoparticles, microcapsules, laminates, matrices and microporous powders (Linhardt,

1988: 53). In all these systems, the drug is merely dispersed or incorporated into the system without the formation of a covalent bond between the drug and the polymer.
The second type consists of polyvinylic or polyacrylic, polysaccharidic and poly(α-amino acid) carriers. In all these systems, the active drug is covalently bonded to the polymeric carrier (Ringsdorf, 1995: 956). The main advantage of using macromolecules as drug carriers is that these molecules are biostable and biodegradable.

Of all the macromolecular drug carriers, the poly(α-amino acids) have significant advantages over other polymers. These type of carriers can be used as possible drug carriers as well as plasma expanders, in order to decrease the required dose, dosage intervals and drug toxicity. Another great advantage of these carriers are their ability to improve a drug's solubility which will lead to better therapeutic efficiency by altering body distribution. This often results in delayed release of the active drug into body fluids which will prolong pharmacological action and will lead to fewer dosage intervals (Giammona *et al.*, 1989: 56, Grcic *et al.*, 1995: 39, Kalcic *et al.*, 1996: 31 and Hoes *et al.*, 1986: 267).

Poly-amino acid carriers can also hold the advantage that these compounds are similar to proteins. It can therefore be assumed that it could be cleaved in the body to amino acids or small peptides, which would easily be eliminated, and also contribute to the patient's nutrition (Neri *et al.*, 1973: 893).

According to Giammona *et al.* (1994: 58) and Grcic *et al.* (1994: 39) poly[α,β-(*N*-2-hydroxyethyl-DL-aspartamide)] (PHEA) is most promising poly(α-amino acid) carrier because it is:

- hydrophilic,
- non-toxic,
- non-antigenic,
- biodegradable in the presence of several enzymes,
- produced easily and at low cost.

PHEA furthermore has a molecular weight of between 10 000 and 90 000, which is high enough to be retained in the blood to exert a steady pharmacological effect (Neri *et al.*, 1973: 893).

A great variety of drugs have already been bonded to PHEA. L-Dopa is one such example. Due to its short plasma half-life, frequent high dosages are necessary to

maintain acceptable therapeutic plasma concentrations. PHEA prodrugs can help to prolong pharmacological activity, minimise unfavourable side-effects and decrease the required dose needed in Parkinson disease patients (Grcic *et al.*, 1994: 39).

PHEA prodrugs can in turn also be used where long-term treatment of a certain drug is required. Gemfibrozil is one such example. This drug is mainly used in the treatment of dyslipidaemias and long-term prevention of atherosclerosis. A macromolecular prodrug of gemfibrozil may offer many advantages compared to other gemfibrozil delivery systems. Increased drug solubility and prolonged drug release can lead to a more convenient drug regimen, as well as increased drug stability and targetability (Lovrek *et al.*, 2000:60).

1.4 PHEA-drug conjugate synthesis

A drug substance containing a terminal carboxylic acid group offers a great number of possibilities for macromolecular prodrug formation. These drugs can be bonded to PHEA as is illustrated in figure 1.6. In the first step the carboxylic acid groups of these drugs react with *N*-1-benzotriazolecarboxylic acid chloride (BtcCl), forming *N*-acyl benzotriazoles, (also called the benzotriazolides). After decarboxylation benzotriazolides (R-COBt) form. These benzotriazolides readily react with hydroxyl compounds, giving rise to polyhydroxyl compounds (in this case PHEA drug conjugate). The reaction proceed under mild conditions under the presence of triethylamine (TEA) as a catalyst (Zorc & Butula, 1994:106 and Zorc *et al*, 1993: 129).

$$R-COOH + BtcCl \xrightarrow[HCl]{TEA} [R-COOBtc] \xrightarrow{-CO_2} R-COBt \xrightarrow[-BtH]{\substack{TEA\\PHEA}} PHEA\ drug\text{-}ester$$

Figure 1.6: The preparation of PHEA prodrugs. Where: TEA = triethylamine, PHEA = poly[α,β-(*N*-2-hydroxyethyl-DL-aspartamide)] and BtcCl = *N*-1-benzotriazolecarboxylic acid chloride (Zorc *et al.*, 1993: 131).

Because the advantages of the macromolecular prodrugs speak for themselves, these type of prodrugs can offer a great number of possibilities. Drugs with short plasma half-life and severe side-effects will benefit the most from a macromolecular prodrug formulation. The NSAIDs are one such group of drugs that is renown for their severe side-effects when used over long-term. The major disadvantages and possible macromolecular prodrug approaches for the NSAIDs will be discussed further in chapter 2.

Chapter 2: NSAIDs: Discussion and study objectives

2.1Introduction

All the NSAIDs (different classes illustrated in table 2.1) are used for their analgesic, anti-inflammatory and anti-pyretic activities. These drugs exhibit the same mechanism of action, which is the inhibition of the cyclooxygenase enzyme responsible for the biosynthesis of the prostaglandins and certain related autacoides. NSAIDs are very effective when used mainly for occasional pain like headache, fever, postoperative pain and other general pains (Insel, 1996: 620).

Table 2.1: Chemical classification of the nonsteroidal anti-inflammatory drugs (Insel, 1996: 621).

Chemical class	Examples
1. Salicylic acid derivatives	Acetylsalisalic acid, sodium salicylate, choline magnesium trisalicylate, salalate, diflunisal, sulfasalzine, olsalazine
2. Para–aminophenol derivates	Paracetamol
3. Indole and indene acetic acids	Indomethacin, sulindac, etodolac
4. Heteroaryl acetic acids	Tolmetin, diclofenac, ketorolac
5. Arylpropionic acids	Ibuprofen, naproxen, fluriprofen, ketoprofen, fenoprofen, oxaprozin
6. Anthranilic acids (fenamates)	Mefenamic acid, meclofenamic acid
7. Enolic acids	Oxicams (piroxicam, tenoxicam), pyrazolidinediones (phenylbutazone, oxyphenthatrazone)
8. Alkanones	Nabumetone

2.2 Advantages and disadvantages of the NSAIDs

With average "everyday" illnesses the NSAIDs show little side-effects (Insel, 1996: 620). It is only with the chronic use of the NSAIDs, for example in the treatment of rheumatoid arthritis and other autoimmune diseases, that the disadvantages of these drugs really become obvious (Insel, 1996: 620). Although all NSAIDs are antipyretic, analgesic and anti-inflammatory, there are still major differences in their activities. For example, paracetamol is antipyretic and analgesic, but poorly anti-inflammatory. These differences are not fully understood, but differential sensitivity of enzymes is one hypothesis (Insel, 1996: 620).

This brings about that only certain NSAIDs can be employed in the long-term treatment of patients with autoimmune illnesses (as illustrated in table 2.2).

Table 2.2: Summary of the NSAIDs that can be used in the long-term treatment of autoimmune illnesses (Hellmann, 1997:770).

NSAID group with examples	Plasma hal life (hours)	Advantages	Disadvantages
Salicylic acid derivatives aspirin	0.25	Inexpensive	Frequent high dosages, (1 g three times a day) Total dose of aspirin needed to achieve improvement varies considerably among individuals. Gastric ulceration
Heteroaryl acetic acids diclofenac tolmetin ketolac	1 5 5	Better tolerated than aspirin.	Gastrointestinal side-effects like epigastric pain, nausea, vomiting, gastric and duodenal ulceration.
Indole and indene acids indomethacin sulindac etolac	3 1 7	Better tolerated than aspirin in patients with autoimmune illnesses.	Severe toxic effects, 35% - 50% of patients receiving usual therapeutic dosages of indomethacin experience untoward symptoms like anorexia, nausea and abdominal pain. Single ulcers or multiple ulcerations of entire upper gastrointestinal tract.
Anthranilic acids mefenamic acid meclofenamate sodium	1 – 4 2 - 4		Not drug of choice for treatment of autoimmune illnesses. Potentially serious haemolytic anaemia.
Propionic acid derivatives ibuprofen naproxen fenoprofen ketoprofen oxaprozin flubiprofen	2 14 3 2 40 6	Better tolerated than aspirin and indomethacin. Reduction in joint swelling, pain and duration of morning stiffness. Improved strength, mobility and stamina. Side-effects is less than that associated with ingestion of high doses of aspirin.	Gastrointestinal intolerance, like epigastric pain, nausea, heartburn – less than that of indomethacin or aspirin.
Enolic acids piroxicam tenoxicam phenylbutazone	48 70 50	Better tolerated than aspirin, indomethacin and naproxen for long-term treatment.	Skin rash Dizziness Heartburn

From the above table it is obvious that some drugs in the same group can have very different plasma half-lives, for e.g. naproxen and oxaprozin with plasma half-lives of 14 and 40 hours respectively, in comparison with ibuprofen and fenoprofen with plasma half-lives of 3 and 2 hours - all four drugs being propionic acid derivatives. Because the propionic acid derivatives are considered as the first line of NSAID treatment for auto-immune illnesses, it is very important to optimise dosages in order to lower the patient's risk to develop serious side-effects (Hellmann, 1997: 669).
If the plasma half-lives of some of these drugs can be prolonged, a greater variety of the NSAIDs could be used effectively in patients with autoimmune illnesses. Daily dosages can be lowered (resulting in less side-effects), with more time between dosages (resulting in better patient compliance).

There are two common problems experienced with long-term usage of the NSAIDs. The first is their severe blood-toxicity. Phenylbutazone is one such example. This drug can cause serious blood disorders like aplastic anaemia, leukopenia, agranulocytosis and trombocytopenia. The usage of phenylbutazone is therefore prohibited in some countries (Hellman, 1997: 771). This drug can therefore definitely not be used over long periods.

The second major problem encountered with the long-term usage of the NSAIDs is gastro-intestinal side-effects. It is estimated that 25% of deaths from peptic ulcer disease annually in the USA resulted from the long-term use of NSAIDs (Hellman, 1997:770). These peptic ulcers are mainly caused by frequent high dosages, which cause patients to quit NSAID treatment, and rather live with the illness than living with the side-effects.

Many different attempts have already been made trying to solve the high incidence of peptic ulcer disease. These attempts resulted in many different approaches, aspirin being one such example. Many different formulations of aspirin are available on the market, including enteric coated aspirin that is very popular because it decreases the high frequency of gastric ulceration seen with the chronic use of plain aspirin (Hellman, 1997:770). These enteric coated tablets can be very useful to lower the incidence of gastro-intestinal symptoms, but will not prolong pharmacological action, needed for better patient compliance.

No matter what the disadvantages of the NSAIDs, they are still generally considered as the first line of treatment against most autoimmune illnesses, and millions of people rely on them everyday. It is therefore important not only to lower gastro-intestinal side-effects, but also prolonging pharmacological action, in order to improve patient tolerance of these drugs.

Macromolecular PHEA prodrugs (as discussed in chapter 1) is one attempt that can be made to improve major disadvantages of NSAID treatment. According to Neri *et al.*, (1973: 893) the clotting time with a poly-amino acid drug carrier can also be reduced, resulting in less blood disorders associated with the long-term use of certain NSAIDs. Furthermore, esterification of acidic drugs can lower or even suppress gastrotoxicity by reducing direct contact between the active drug and the gastro-intestinal mucosa (Whitehouse *et al.*, 1980: 795 and Jardine *et al.*, 1995: 960). Poly-amino acid drug carriers can also improve site specificity. By targeting the pathological process, damage to healthy tissue can be minimised resulting in less side-effects (Kopecek *et al.*, 1987: 315 and Zorc *et al.*, 1995: 65).

Investigations conducted by Giammona *et al.*, (1994: 58) have also shown that the interaction between some of these poly-amino acid prodrugs, chosen as biomembrane models, showed that the polymer-drug conjugates interact with these systems more strongly than the free drugs. Moreover *in vivo* pharmacological tests have shown that poly-amino acid NSIA prodrugs show analgesic and anti-inflammatory activities comparable to that of the free drug, which indicates that the free drug is completely released from the carrier.

The propionic acid derivatives are widely regarded as first line of treatment when long-term NSAID treatment is started. They are especially effective in the treatment of all types of auto-immune illnesses and offer great advantages over other NSAIDs like aspirin and indomethacin. Because all the propionic acid derivatives contain a terminal carboxylic acid group, these drugs offer a great number of possibilities for prodrug formation. This diverse group of NSAIDs will be further discussed in chapter 3.

2.3 Study objectives

Because the advantages of the macromolecular prodrugs speak for themselves, it is worth trying to formulate some of the NSAIDs most commonly used over long-term into macromolecular prodrugs. If these drug carriers can be used to prolong pharmacological

action and lower drug toxicity, some of the older NSAIDs (with limited usage at present) can again be used.

The aim of this study was therefore

- to determine the relevant physical properties influencing the analysis of the conjugate,
- to prove that fenoprofen and PHEA really are chemically bound to each other and that fenoprofen is not just merely dispersed or incorporated into PHEA,
- to develop a HPLC and a spectrophotometric method for the analysis of a PHEA-fenoprofen conjugate and to compare these two methods with each other,
- to fully validate the developed HPLC method,
- to determine fenoprofen drug loading in the conjugate by means of hydrolysis,
- to determine and describe the kinetics of fenoprofen release from the conjugate,
- to determine the release of fenoprofen from the conjugate under certain simulated biological conditions.

The above mentioned information can proof to be very useful in future to assist in the day to day analytical determination of this PHEA-fenoprofen conjugate, should it ever become therapeutically applicable.

Chapter 3: Propionic acid derivatives

3.1 Introduction

The propionic acid derivatives (illustrated in figure 3.1) represent a group of effective and useful NSAIDs. They offer significant advantages over other NSAIDs, since they are better tolerated (Green *et al.*, 1993: 386). The approved indications for the use of the propionic acid derivatives include the symptomatic treatment of rheumatoid arthritis, osteoarthritis, ankylosing spondylitis and acute gout arthritis. They can also be used as analgesics, for acute tendinitis and bursitis. In the long-term treatment of rheumatoid arthritis and other auto-immune illnesses, there is a reduction in joint swelling, pain and duration of morning stiffness. Strength mobility and stamina are improved (Hellman, 1997: 770).

Table 3.1: The propionic acid derivatives (Sneader, 1986: 94).

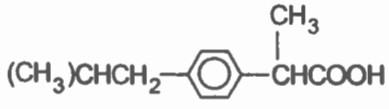
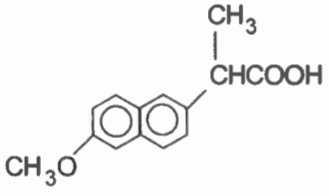
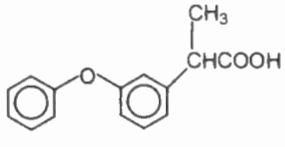
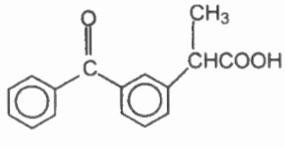
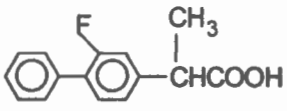
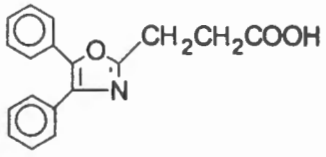
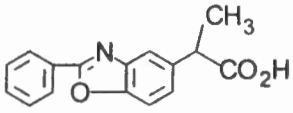
Drug name	Structure	Plasma half- life
Ibuprofen		2 hours
Naproxen		14 hours
Fenoprofen		3 hours
Ketoprofen		2 hours

Table 3.1 (continued): The propionic acid derivatives (Sneader, 1986: 94).

Drug name	Structure	Plasma half- life
Flurbiprofen		6 hours
Oxaprosin		40- 60 hours
Benoxaprofen		Taken off the market in 1983 because of accumulation of high drug levels in elderly patients.

The differences in the pharmacological properties of the propionic acid derivatives are striking. Not only does the plasma half-lives of these drugs range from 2 to 60 hours, but their potencies and gastro-intestinal side-effects also differ dramatically.

The propionic acid derivatives all contain a chiral centre and they are administered as racemates. The inactive (R)-enantiomers of these drugs undergo chiral inversion, but the rate of chiral inversion differs from drug to drug. This is why these drugs differ from each other in potency and toxicity (Lennard *et al.*, 1990: 196).

Stereoselective metabolism is a major determinant of the pharmacokinetics of the propionic acid derivatives. For example, the metabolism of (S)-ibuprofen is much slower than that of (R)-ibuprofen. High plasma concentrations of (S)-ibuprofen were observed after dosing only with (R)-ibuprofen. When given high dosages of (S)-ibuprofen, very little to no (R)-ibuprofen was found in the plasma. This hypothesis was supported by *in vitro* studies, showing that (R)-ibuprofen binds more avidly to human plasma than (S)-ibuprofen (Lennard *et al.*, 1990: 196).

This phenomenon is true for all propionic acid derivatives. This implicates that one propionic acid derivative can be more potent, with longer plasma half-life, because the rate of chiral inversion is quicker. The shorter the rate of chiral inversion, the less inactive enantiomer present in the urine, which will result in more active drug binding to human plasma (Lennard *et al.*, 1990: 196).

Stereoselective metabolism of the propionic acid derivatives may be linked to the development of side-effects like blood disorders. This finding can be explained through strong evidence that the inversion of the (R)-enantiomer to (S)-enantiomer proceeds through the stereospecific formation of the coenzyme A thioester of the (R)-enantiomer. Studies have indicated that the coenzyme A esters of the (R)-enantiomer can be incorporated into triacylglycerols in membranes, by replacing natural fatty acids. These incorporated triacylglycerols are eliminated very slowly and may accumulate leaving long-term residues that may interfere with normal lipid metabolism and membrane function, giving rise to the toxic effects experienced with the propionic acids (Lennard *et al.*, 1990: 197).

As a result of the toxicological implications of stereospecific coenzyme A thioester formation, there may be clinical advantages in administering the pure (S)-enantiomer rather than the racemic mixture of the propionic acid derivatives (Lennard *et al.*, 1990: 197). The use of only the (R)-enantiomer has been suggested to minimise gastro-intestinal side-effects, but holds no real advantage because gastro-intestinal ulceration is not only caused locally, but also systemically (Lennard *et al.*, 1990: 198). It can therefore be seen that macromolecular prodrugs can also hold major advantages for the propionic acid derivatives.

The propionic acid derivatives like naproxen and oxaprozin are more popular for long-term treatment of auto-immune illnesses than ibuprofen or fenoprofen with shorter plasma half-lives. These propionic acid derivatives with longer plasma half-lives are better tolerated and more convenient to take (due to longer time intervals between dosages). Because patients and doctors prefer these drugs, propionic acid derivatives with shorter half-lives are not widely used anymore. Although these drugs can also be very effective over long-term, they tend to cause more side-effects than there counterparts with longer plasma half-lives.

Fenoprofen is another propionic acid derivative useful in the treatment of auto-immune illnesses. But because of short plasma half-life (3 hours), fenoprofen requires frequent high dosages (300-600 mg three to four times daily), which in the past resulted in severe gastro-intestinal and blood toxicity (Insel, 1996: 625). Due to this reason fenoprofen is not widely used anymore, with only one fenoprofen preparation (Fenopron®) available on the South African market at the moment.

A macromolecular (PHEA) prodrug of fenoprofen can possibly improve fenoprofen's therapeutic value. This will mean that patients who do not respond well to other NSAIDs, may have a better choice from more drugs to use for their day to day NSAID needs. Furthermore the side-effects encountered with these drugs can be less. Dosage intervals will be longer, and the drugs will

be better tolerated over long term. Even drug costs can be lowered, because synthesis of these drugs are far less costly than some newer NSAIDs on the market.

3.2 Propionic acid derivatives: fenoprofen

Fenoprofen is produced as a calcium dihydrate salt [2-(3-phenoxy-phenyl)propionic acid calcium salt] (figure 3.2), and is a racemic mixture, with no apparent difference between the pharmacological activities of the (R) and (S) isomers and the racemate (Brooks *et al.*, 1996: 71).

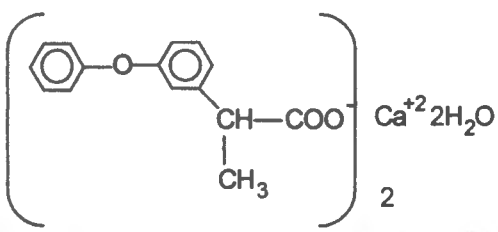


Figure 3.2: Calcium dihydrate salt of fenoprofen (Brooks *et al.*, 1996: 71).

3.2.1 Synthesis

The synthesis of fenoprofen is presented in figure 3.3. Acetophenone is reduced by sodium borohydride to the corresponding 1-arylethanol, and then reacted with phosphorus tribromide to give the 1-arylethyl bromide. Nucleophilic exchange with sodium cyanide gives 2-arylpropionitrile which is hydrolysed to fenoprofen (Roth *et al.*, 1988: 100).

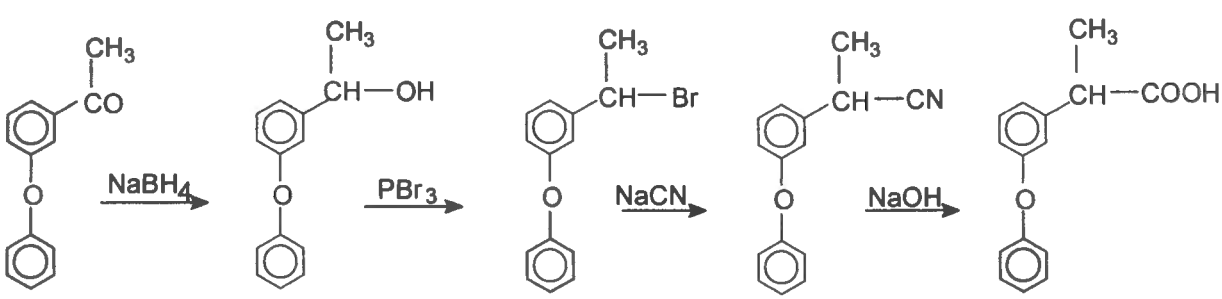


Figure 3.3: Synthesis of fenoprofen (Roth *et al.*, 1988: 100)

3.3.2 Physical properties

3.3.2.1 Physical appearance

Fenoprofen is an odourless, white, crystalline powder (Ward & Schirmer. 1977: 165).

3.2.2.2 Melting range and differential thermal analysis

When an open pan thermogram (illustrated in figure 3.4) of fenoprofen calcium is run, a large endotherm appears near 94 °C, which corresponds to a loss of water accompanied by collapse of the crystal structure. Fenoprofen melts at 118-123 °C and is accompanied by partial liquification (Ward & Schirmer, 1977: 165).

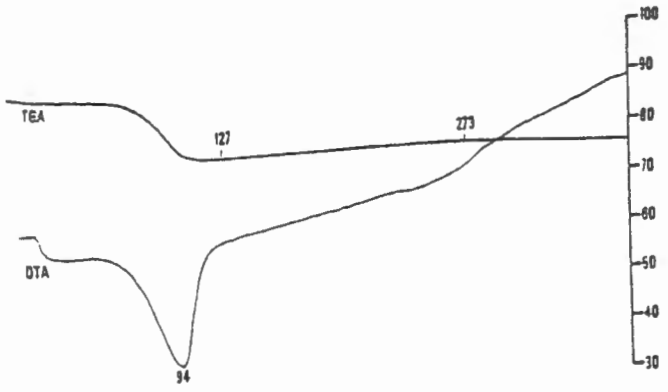


Figure 3.4: Thermogram of fenoprofen calcium (Ward & Schirmer., 1977: 165)

3.2.2.3 Dissociation constant

Fenoprofen calcium is a weak acid with a dissociation constant (pK_a) of 4.5 at 25 °C (Ward & Schirmer, 1977: 166). This means that fenoprofen calcium will reach equilibrium between the ionised and non-ionised species at a pH of 4.5. Fenoprofen's solubility will therefore be greater at pH values higher than 4.5.

3.2.2.4 Solubility

Table 3.2: Solubility of fenoprofen calcium in different solvents (Ward & Schirmer, 1977: 166).

Solvent	Solubility (mg/ml)	Temperature (°C)
Methanol	8.00	37
1- Hexanol	11.00	37
Chloroform	0.01	37
Cyclohexane	~0.01	37
Water	2.50	25
Buffer pH 1.2	0.12	25
pH 4.0	0.28	25
pH 6.0	3.30	25

3.3.2.5 Ultraviolet absorption spectrum

The ultraviolet spectrum of fenoprofen calcium in methanol is shown in figure 3.5. The spectrum exhibits maxima at 266, 272, and 278 nm (Ward & Schirmer., 1977: 166). In aqueous acid, maximum absorption is exhibited at 272 nm, with $A_{1cm}^{1\%} = 72$. In methanol, maximum absorption is exhibited at 273 nm, with $A_{1cm}^{1\%} = 72$ (Moffat *et al.*, 1986: 615).

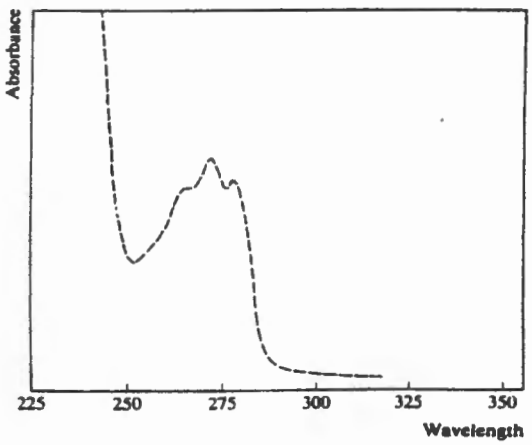


Figure 3.5: The ultraviolet spectrum of fenoprofen calcium (Moffat *et al.*, 1986: 615)

3.3.2.6 Infrared spectrum

The infrared spectrum of fenoprofen calcium is given in figure 3.6. Major band assignments are shown in table 3.3.

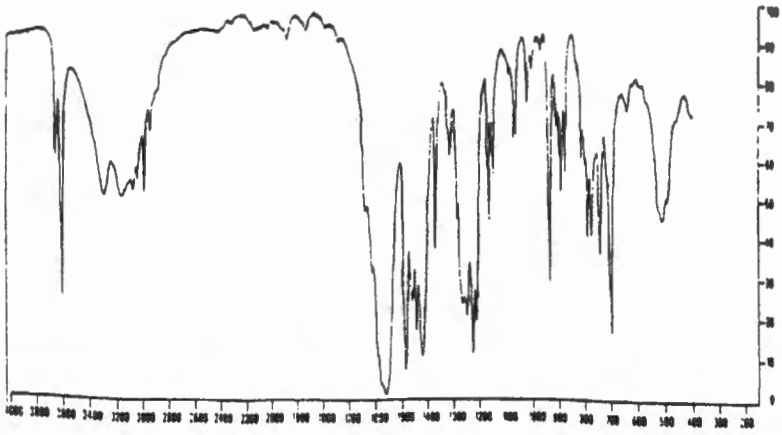


Figure 3.6: Infrared spectrum of fenoprofen calcium (Ward & Schirmer, 1977: 167).

Table 3.3: Major band assignments.

Band position (cm^{-1})	Assignment
3660, 3600 and 3300	-OH stretching of hydrate
1560 (very strong, broad) and 1420	COO^- asymmetric and symmetric stretching
1490, 1440 and 1450	Aromatic ring stretching
1260 to 1210 (several bands)	C – O – C asymmetric ether stretching
930 to 695 (several bands)	primarily aromatic out of plane bending

3.2.2.7 Nuclear magnetic resonance spectrum

The 60 MHz proton NMR spectrum of fenoprofen calcium in deuterated dimethylsulfoxide acidified with trifluoracetic acid is shown in figure 3.7. Assignments of the signals are given in table 3.4.

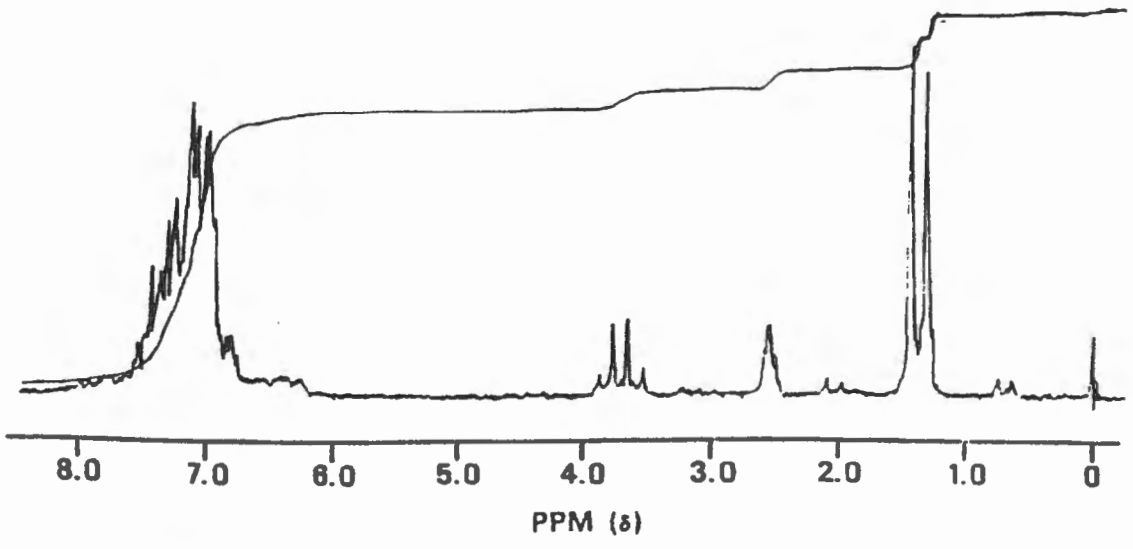


Figure 3.7: ^{1}H NMR spectrum of fenoprofen calcium (Ward & Schirmer., 1977: 169).

Table 3.4: Assignments of the ^{1}H NMR signals of fenoprofen calcium (Ward & Schirmer., 1977: 167).

Signal ppm	Assignment
8.4-6.0 (complex multiplet)	Aromatic protons
3.70 (quartet, J = 7 Hz)	- CH -
1.35 (doublet, J = 7 Hz)	- CH_3

3.2.2.8 Mass spectrum

The mass spectrum of fenoprofen is presented in figure 3.8. (Relative molecular mass of fenoprofen is 242.3, in comparison with 558.60 of fenoprofen calcium dihydrate).

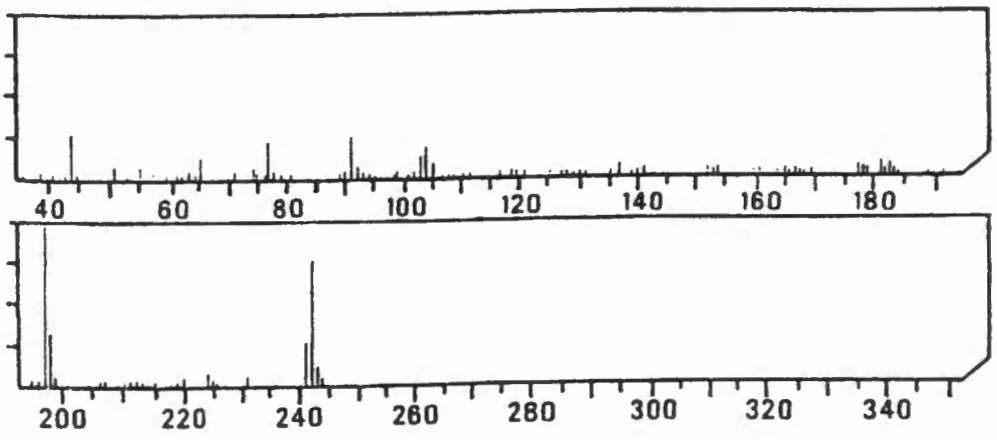


Figure 3.8: The mass spectrum of fenoprofen (Ardrey, *et al.*, 1985: 258)

3.3.2.9 Stability

Fenoprofen calcium is quite stable in acid, base and heat. Storage at 135 °C for six days results only in loss of water of hydration. Samples stored for three years at 37 °C showed no degradation. Exposing aqueous solutions of the drug to intense ultraviolet light can induce degradation of fenoprofen calcium. Under these conditions the photo-Fries rearrangement occurs, leading to a mixture of the isomeric biphenyls shown in figure 3.9 (Ward & Schirmer, 1977: 172) and (Budavari *et*, 1989: 3929). No degradation of fenoprofen calcium has been observed in any pharmaceutical formulations over a period of three years at 37 °C. In preformulation studies fenoprofen was found to be stable at relative humidities in the range of 1 % to 98 % at 25 °C (Ward & Schirmer, 1977: 172).

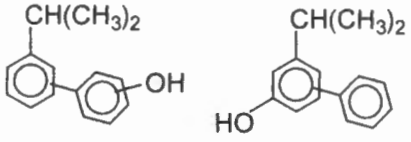


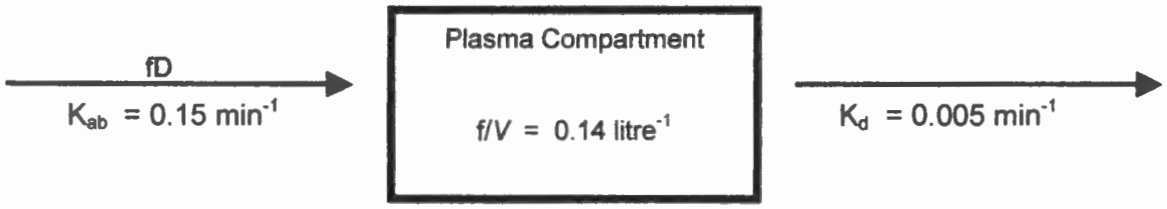
Figure 3.9: Isomeric biphenyls of fenoprofen after ultraviolet exposure (Ward & Schirmer, 1977: 172).

3.3. Pharmacological aspects

3.3.1 Pharmacokinetics

Oral doses of fenoprofen are quickly but incompletely (85 %) absorbed. The presence of food in the stomach lowers drug absorption and lowers peak plasma concentration in plasma. Peak plasma concentration is usually reached within 2 hours after oral dosing. After absorption, fenoprofen is almost completely (99 %) bound to plasma albumin.

A one compartment open model provides a reasonable accurate description of fenoprofen concentration in plasma after oral doses. Kinetic parameters for the one compartment model is given in figure 3.10 (Ward & Schirmer, 1977: 165). Renal clearance values for fenoprofen range from 38.6 to 47.8 ml/ min, which suggest tubular resorption (Ward & Schirmer, 1977: 174).



K_{ab} = absorption rate constant

K_d = elimination rate constant

f = fraction of dose absorbed

fD = fraction of dose absorbed

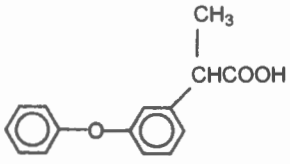
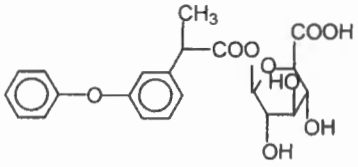
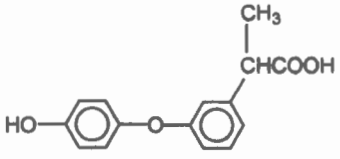
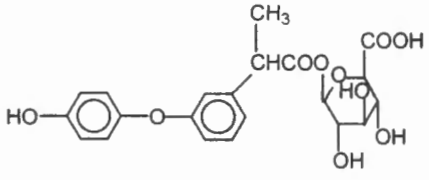
V = volume of the plasma compartment

Figure 3.10: The one compartment open model for fenoprofen concentrations in the plasma following oral doses (Ward & Schirmer, 1977: 175).

3.3..2 Metabolism

Metabolism of fenoprofen calcium involves hydroxylation of the terminal phenyl group and conjugation with glucuronic acid. The drug is extensively (> 90 %) metabolised and excreted almost entirely in the urine (Insel, 1996: 640). The different metabolites in human urine are shown in table 3.5 (Ward & Schirmer, 1977: 174).

Table 3.5: The different metabolites of fenoprofen calcium in human urine (Ward & Schirmer., 1977:174).

Type of metabolite	Metabolite structure	Percentage present in human urine
(I) (Unchanged fenoprofen)		3%
(II)		45%
(III)		2%
(IV)		42%
(V)	Unidentified acid labile conjugate	3 %
(VI)	Unidentified acid labile conjugate	5 %

3.3.3 Mechanism of action

Because the pathogenesis of some auto-immune illnesses (like rheumatoid arthritis) is still unknown, these diseases appear to be activated primarily by activated T cells, giving rise to T cell-derived cytokines, such as interleukin 1 (IL-1), and tumor necrocis factor (TNF). Although activation of B cells and humoral response are evident, most of the antibodies generate IgG. Cytokines like IL-1 and TNF have been found in the rheumatoid synovium, although some of the actions of these cytokines are accompanied by the release of prostaglandins and/or thromboxane A_2 and only their pyrogenic effects are blocked by inhibitors of cyclooxygenase (Insel, 1996: 619).

The main mechanism of action of fenoprofen and other NSAIDs is the inhibition of cyclooxygenase enzyme responsible for the biosynthesis of prostaglandins and certain related autacoides. It is therefore difficult to ascribe the antirheumatoid effects of the NSAIDs to

inhibition of prostaglandin synthesis. It has been proposed that salicylate and certain other NSAIDs can directly inhibit the activation and function of the above neurophils, perhaps by inhibition of membrane-associated processes. These processes are independent of these drug's ability to inhibit prostaglandin synthesis (Insel, 1996: 620).

3.3.4 Toxic effects

The most frequently reported side-effects of fenoprofen are gastro-intestinal ones. This includes abdominal discomfort and dyspepsia. These side-effects are almost always less than that of equivalent doses of aspirin. Other side-effects include skin rash, tinnitus, dizziness, lassitude, confusion and anorexia (Insel, 1996: 640).

3.3.5 Indications

Fenoprofen is indicated for the symptomatic treatment of rheumatoid arthritis, osteoarthritis, anklosing spondolytis, acute gout, acute tendinitis and bursitis (Insel, 1996: 638).

3.4 PHEA-fenoprofen conjugate

3.4.1 Synthesis

PHEA-fenoprofen conjugate can be prepared according to the method described in paragraph 1.4 and illustrated in figure 3.11. In the first step 1-benzotriazole carboxylic acid chloride (BtcCl) is dissolved in toluene and is dropwise added to a solution of fenoprofen, triethylamine and toluene. The reaction mixture is stirred for 2 hours at room temperature and extracted three times with water. The organic layer is dried over anhydrous sodium sulfate and evaporated. A mixture of ether and petrolium is added to the residue and the pure fenoprofen benzotriazolide is filtered off. PHEA-fenoprofen conjugate is prepared by adding a solution of PHEA, benzotriazotriazolide and triethylamine in of dimethylformamide (DMF). This mixture is left for three days at room temperature with occasional shaking. The solvent is then evaporated in vacuo to a small volume. The polymeric product (PHEA-fenoprofen conjugate) is precipitated by adding acetone and the product filtered off and washed several times with a small amount of acetone until benzotriazole is completely removed (Zorc *et al.*, 1993: 128, and Zorc *et al.*, 1994: 104).

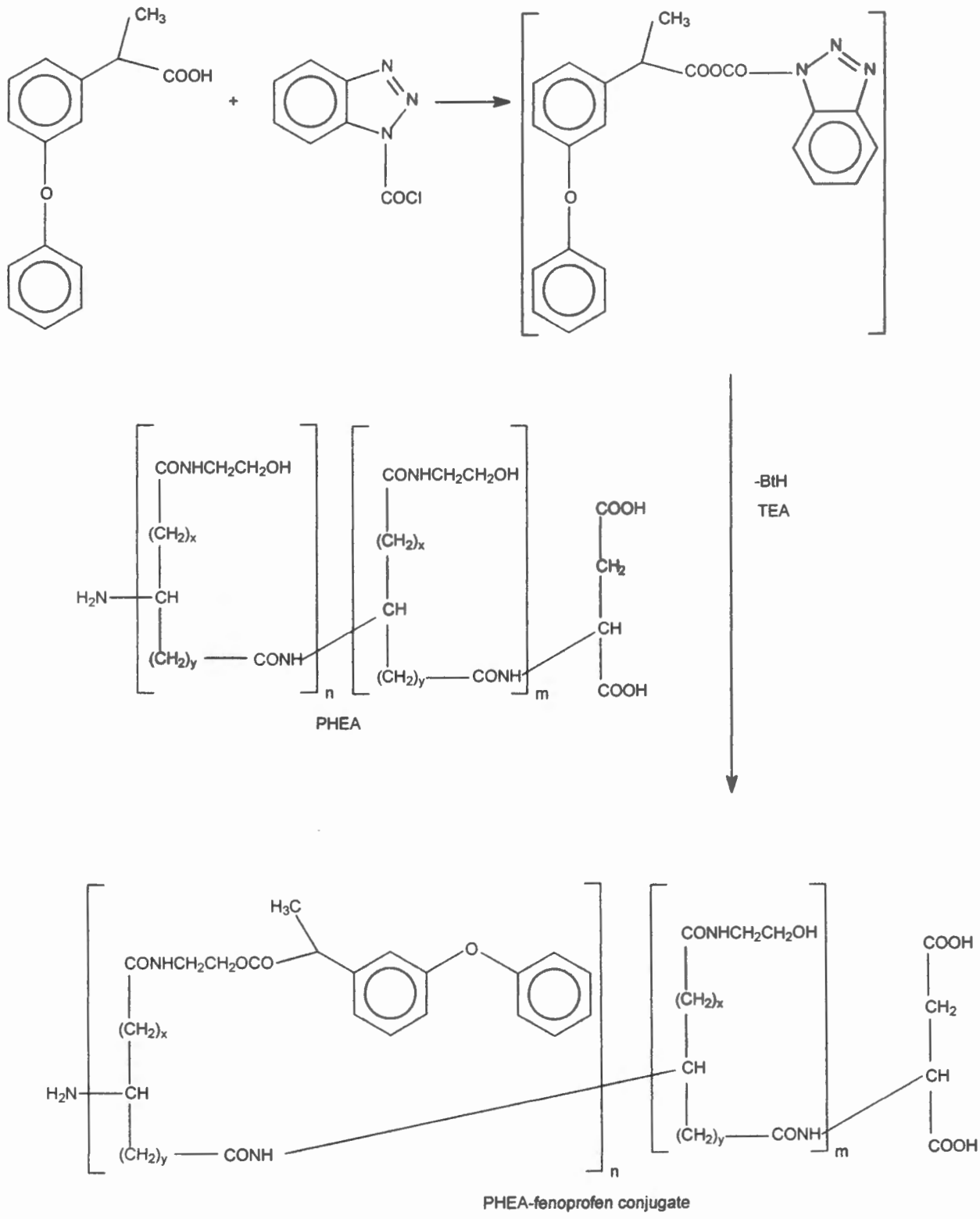


Figure 3.11: The synthesis of fenoprofen-PHEA drug ester (Zorc, 1994: 104).

Chapter 4: Method validation: Terminology

4.1. Introduction

Method validation is the process to confirm that the analytical procedure employed for a specific test is suited for its intended use (Huber, 1998: 3). Methods that need to be validated are those methods who will be introduced for routine use. If conditions change outside the original scope for which the method has been validated, it is important that validation must be repeated (Huber, 1998: 3).

The importance of analytical validation is portrayed in the considerable amount of attention it receives in literature, industrial committees and regulatory authorities. The current Good Manufacturing Practice (cGMP) regulations require test methods used to assess compliance of pharmaceutical products to meet proper standards of accuracy and reliability (Brittain, 1997: 275).

The Food and Drug Administration (FDA) published a final guideline on the validation of analytical procedures on 1 March 1995. The contents of the guidelines were prepared with the help of the International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human use (Brittain, 1997: 275).

According to section 501 of the USA Federal food, drug and cosmetic act, assays and specifications in monographs of the Unites States Pharmacopoeia and the National Formulary comply with legal standards. As a result every analytical method should be validated according to the current Pharmacopoeia standard (Brittain, 1997: 275).

Method validation should not be seen as a one-time situation to fulfil certain rules and regulations, but the methods should be continuously validated and should also be designed by the developer or user to ensure ruggedness and robustness. The methods should be reproducible when used by other analysts and other equivalent equipment on different days or locations, and throughout the duration of the life of the drug in question. Any data generated for acceptance release, e.g. stability or pharmacokinetic studies, will only be trustworthy if the method that generated the data was reliable. Validation should also be done as soon as possible in the development of a new method, before important data can be generated. Validation should also be an ongoing process

in the form of re-validation with any method changes that may occur (CMCCC of the CDER, 1994: 1).

It is also important to notice that many different approaches may be applicable and acceptable. It is the responsibility of the developer to choose the validation procedure and protocol most suitable for the situation. The main objective of validation of an analytical procedure is to demonstrate that the procedure is suitable for its intended use (CMCCC of the CDER, 1994: 1).

4.2. Assay types

The USP (2000: 2150) general tests allow for differentiation between assay types, with the decision on the required performance parameters needed for their validation, (summary of the various assay categories and their required validation parameters are given in table 4.1).

Three categories are distinguished:

- Category 1: Assays are developed for the quantitation of major components of bulk drug substances or active ingredients.
- Category 2: Assays are used for the determination of impurities in bulk drug substances or degradation products.
- Category 3: Assays are used to determine performance characteristics such as dissolution profile, disintegration time, rate of drug release of a active substance in the dosage form (Brittain, 1997: 275).

If the need for validation for a specific method is not clear, there is an underlying principle that should be followed: "When in doubt, perform the validation" (Brittain, 1997: 276).

Even if an official pharmacopoeia method was adapted, a complete validation is still necessary to ensure that the documented method is sufficiently complete to meet the required experimental needs for this study. It is important to take into account that parameters like linearity, range and detection limits must comply with a specific laboratory and that validation must comply with the specific laboratory environment.

Table 4.1: Various assay categories and required validation parameters (Brittain, 1997: 276).

Analytical performance parameter	Category 1	Category 2	Category 2	Category 3
	Assay	Assay, Quantitative	Assay, Limit Test	Assay
Precision	Yes	Yes	No	Yes
Accuracy	Yes	Yes	Possibly	Possibly
Specificity	Yes	Yes	Yes	Possibly
Limits of detection	No	No	Yes	Possibly
Limits of quantitation	No	Yes	No	Possibly
Linearity	Yes	Yes	No	Possibly
Range	Yes	Yes	Possibly	Possibly
Ruggedness	Yes	Yes	Yes	Possibly

Analytical performance parameters to be evaluated are:

- precision
- accuracy
- specificity
- limits of detection and limit of quantitation
- linearity
- range and
- robustness (Brittain, 1997: 275, Huber, 1998: 7 and CMCCC of the CDER, 1994: 8).

4.2.1 Precision

4.2.1.1 Definition

The precision of an analytical procedure defines the measure of the degree of repeatability between a series of results obtained from multiple samplings of the same homogeneous sample (USP, 2000: 2150, CMCCC of the CDER, 1994: 8, Brittain, 1997: 275, and Huber, 1998: 3).

These samples must be taken under exact conditions required for performance of the method. This quantity is expressed in terms of the relative standard deviation obtained during a multiple series of measurements.

A well-validated method will also include intermediate precision. Intermediate precision is where the degree of repeatability is measured under normal operating conditions. Different analytical laboratories, different instruments and different days can be used to perform intermediate precision validation.

4.2.1.2 Determination

The usual practice is to make at least 6 determinations of analyte (Brittain, 1997: 275, Huber, 1998: 3 and ICH, 1996: 1). Six aliquots of a homogenous sample must be taken for calculation of the relative standard deviation (USP, 2000: 2150 and Brittain, 1997: 275).

For drug analysis in pharmaceutical quality control precision below 2 % is acceptable, while a percentage relative standard deviation (% RSD) of less than 1 % should be easy to achieve (Huber, 1998: 3).

4.2.2 Accuracy

4.2.2.1 Definition

The accuracy of an analytical method is the extent to which test results generated by the method and the true value agrees (Huber, 1998: 3 and Brittain, 1997: 276).

4.2.2.2. Determination

Accuracy is determined by the assay of a known quantity of analyte spiked into a sample matrix within the proper concentration range spanning the anticipated result (USP, 2000: 2150 and Huber, 1998: 12).

It is then calculated as the percentage recovery from the assay of the known spiked amount of analyte in the sample (USP, 2000: 2150). Accuracy should be assessed using a minimum of 6 determinations over a minimum of 3 concentration levels (Brittain 1997: 276).

4.2.3 Specificity

4.2.3.1 Definition

The ability to assess an analyte unequivocally in the presence of other components that might be expected to be present, is defined as specificity (USP, 2000: 2150, (Brittain,

1997: 276, CMCCC of the CDER, 1994: 17). The analyte should have no interference from other extraneous components and must be well resolved from them (Brittain, 1997: 275). Specificity is also necessary to prove the absence of impurities, or that the level of impurities present, is at acceptable levels.

4.2.3.2 Determination

The bias between the pure sample and the spiked samples are determined by calculating the percentage agreement between the two using the equation below (Brittain, 1997: 277).

% Agreement = $(T_P / T_A) \times 100$
where:
T_P = test result in the presence of spiked fenoprofen
T_A = test result in pure fenoprofen samples (Brittain, 1997: 277).

In pharmaceutical specificity validation a %RSD of less than 2 % is acceptable (Huber, 1998: 8).

4.2.4 Linearity

4.2.4.1 Definition

The linearity of an analytical method is its ability to prove that test results are directly proportional to the concentration of analytes in sample within a given range (USP, 2000: 2151), Brittain, 1997: 278 and Huber, 1998: 13). Linearity is determined by a series of three to six standard concentrations whose concentration range between 80 - 120 % of the expected concentration range. The response should be directly or by means of a well defined mathematical calculation, proportional to the concentrations of the analytes (Brittain, 1997: 277).

4.2.4.2 Determination

Linearity is evaluated graphically in most cases (Huber, 1998: 13). The evaluation is made by visual inspection of a plot of response versus concentration of analyte. A linear regression equation applied to the results should have an intercept that is not zero (Huber, 1998: 13). A non-zero intercept demonstrates that there is no effect on the accuracy of the method (Huber 1998: 14).

The slope of the regression line provides the mathematical relationship between the test results and the analyte concentration (Huber, 1998: 3). Linearity can also be estimated by the correlation coefficient (USP, 2000: 2151). The correlation coefficient should be as close to 1.000 as possible. Any method with a correlation coefficient less than 0.99 or more than 1.01 may be insufficiently precise or non-linear (Brittain, 1997: 278).

4.2.5 Limit of detection and limit of quantitation

4.2.5.1 Definition

The limit of detection (LOD) is defined, as the lowest concentration of an analyte in a sample that can be detected, not quantitated (USP, 2000: 2150, CMCCC of the CDER, 1994: 8).

The limit of quantitation (LOQ) is defined as the lowest concentration of an analyte that can be determined with acceptable precision and accuracy under the stated operational conditions of the method (USP, 2000: 2151). It is also expressed as a concentration with precision and accuracy of the measurement also reported

4.2.5.2 Determination

The limits of detection and quantitation of an analytical method are determined by analysing a number of low concentration samples. The concentration of these samples should span the lowest quarter of the range established during the linearity study (Brittain, 1997: 278). Each sample is assayed six times, and the standard deviation of the peak area calculated. This standard deviation and the slope of the calibration curve can then be used to calculated LOQ and LOD mathematically (paragraph 5.5.6).

Limit of detection (LOD) validation is necessary to calculate the lowest concentration of an analyte in a sample that can be detected. Limit of quantitation (LOQ) is necessary to calculate the lowest concentration of an analyte in a sample that can be quantitated with acceptable precision and accuracy.

4.2.6 Range

4.2.6.1 Definition

The range of an analytical method is the interval between the upper and lower levels of analyte concentration for which linearity was proven and spans the concentration of interest (Brittain, 1997: 279 and Huber, 1998: 14).

4.2.6.2 Determination

If it was demonstrated that the method had acceptable precision, accuracy and linearity, the method also complies with acceptable range (Brittain, 1997: 279).

4.2.7 Robustness

4.2.7.1 Definition

Robustness is the capacity of a method to remain unaffected by small deliberate variations in method parameters (Brittain, 1997: 280).

Typical variations in HPLC can be the following:

- pH of the mobile phase
- variations in mobile phase composition
- different columns (different suppliers and batches)
- temperature
- flow rate (ICH, 1996: 10).

The evaluation of robustness of a given analytical method is an integral part of validation. Theoretically, it provides an indication of the reliability of the method during normal usage (Huber, 1998: 15).

4.2.7.2 Determination

Robustness must be done very early in the validation of a new method, so that critical parameters can be identified and adequately controlled. In these cases a precautionary statement must be included in the method documentation (Huber, 1998: 15).

After deciding what parameter to change (like pH of the mobile phase), several injections of each pH are made within the concentration range of the given analyte. Percentage recovery is then used to determine if there are significant changes in the method's effectiveness.

4.3. Conclusion

Methods should not be validated as a one-time situation, but should be validated and designed by the developer or user for a specific laboratory, with specific conditions and equipment. A well-designed method validation will ensure repeatable, accurate and reliable results.

Chapter 5: Reaction kinetics: Definition and theory

5.1 Introduction

Chemical reactions are processes in which a substance or substances are transformed into other substances (Cartensen, 1990: 17). Chemical kinetics is concerned with the analysis of the dynamics of chemical reactions. The rate of a chemical reaction is expressed as a change in concentration of some species with time. The rate of a chemical reaction is therefore given in concentration (House, 1997: 2 and Logan, 1998: 3). Consider hypothetical reaction 5.1 that is taking place in a closed space (House, 1997: 2).

$$A \rightarrow B \qquad (5.1)$$

The reaction rate of reaction 5.1 can either be described as the disappearance of A or appearance of B. To express the rate of change equation 5.2 may be written (Logan, 1998: 3 and House 1997: 2).

$$\text{Rate of reaction} = -d[A]/dt = d[B]/dt \qquad (5.2)$$

The mathematical equation relating concentrations and rate is called the "rate equation law" and can be illustrated in figure 5.1

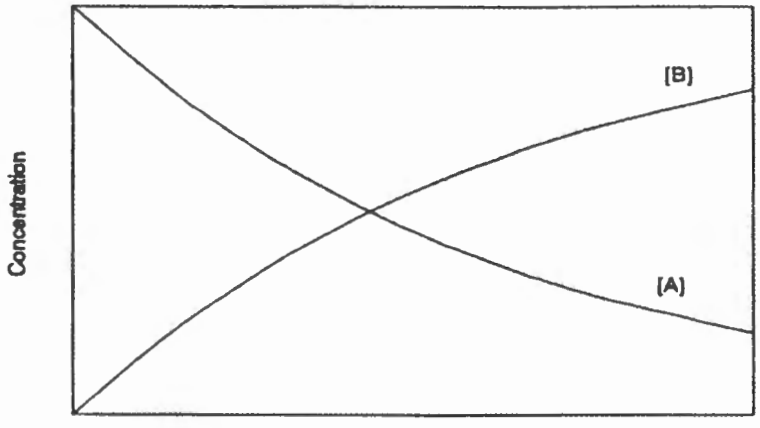


Figure 5.1: Change in concentration of A and B if A → B (House, 1997: 2).

Consider reaction 5.2 (House, 1997: 2):

$$aA + bB \rightarrow cC + dD \qquad (5.3)$$

where:
a, b, c and d are the concentrations of A, B, C and D respectively.

Equation 5.3 can be written as follows (5.4):

$$Rate = k[A]^x[B]^y \qquad (5.4)$$

where:
x and y are the exponents of the concentrations of A and B respectively (the order of the reaction).
k = the rate constant

The overall rate of a reaction is the sum of exponents x and y. These exponents must be established by studying the reaction using different initial concentrations of A and B. The order of a reaction can be:

- zero-order
- pseudo zero-order
- first-order
- pseudo first-order
- second-order
- third (or higher)-order

5.2 Zero-order reactions

Consider hypothetical reaction 5.5 (Ritchie, 1996: 5).

$$A \xrightarrow{k_0} B \qquad (5.5)$$

The rate of a chemical reaction is of zero-order if it is independent of the concentration of the participating substances. If the rate of a reaction is independent of the concentration of the reacting substance A, then the amount d[A] (with decreasing concentration of A) at any given time dt is constant throughout the course of the reaction, and can be given as equation 5.6 (Capellos and Bielski, 1972: 7).

$$-d[A]/dt = k_0 \quad (5.6)$$

where k_0 is the velocity constant with units moles per litre per second (M sec^{-1}) (House, 1997: 6).

The negative sign in equation 5.6 shows that A is removed from the system. If [A] is the concentration of A at time-interval t_1 and $[A]_0$ is the concentration of A during time-interval t_2, equation 5.6 can be written as follow in equation 5.7.

$$k_0 = [A]_0 - [A] / (t_2 - t_1) \quad (5.7)$$

If $t_1 = 0$, equation 5.7 can be written as equation 5.8.

$$[A] = [A]_0 - k_0 t_2 \quad (5.8)$$

A plot of [A] versus time (illustrated in figure 5.2) yields a straight line with $[A]_0$ the intercept and k_0 the slope.

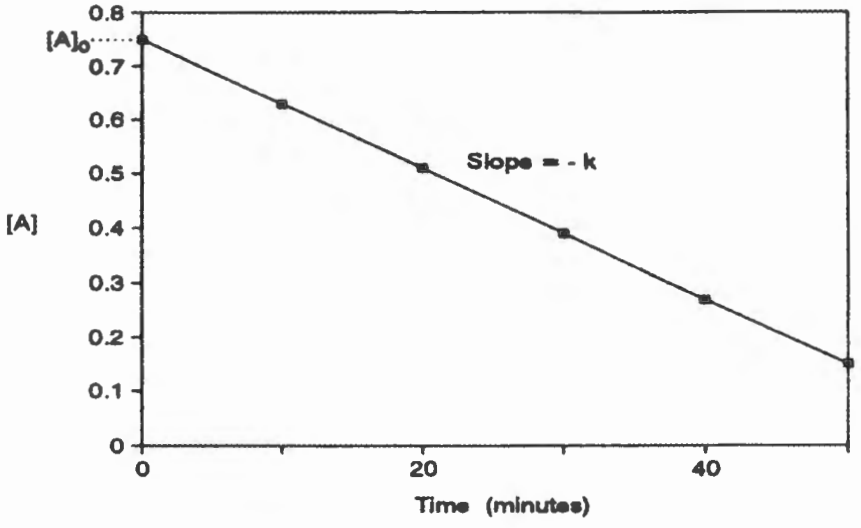


Figure 5.2: Zero-order plot for A → B (House, 1997: 7).

5.2.1 Half-life determination for a zero-order process

Instead of the rate constant half-life ($t_{\frac{1}{2}}$) is often used. This quantity is more tangible since it tells how much time is required for the concentration of A to fall to one-half of its value from the initial measurement. The half-life of a zero-order reaction can be determined because $[A] = [A]_0 - kt_{\frac{1}{2}}$, $t_{\frac{1}{2}}$ can therefore be written as equation 5.9 (House, 1997: 5).

$$t_{\frac{1}{2}} = [A]_0 / 2k \quad (5.9)$$

5.2.2 Pseudo zero-order reactions

This type of rate law is not especially common, and is followed by some reactions where some factor governs the reaction rate (House, 1997: 11). Zero-order reactions are independent of the concentration of the reacting substance A, and with time (dt) the concentration of A decreases and is constant throughout the course of the reaction (Capellos and Bielski, 1972: 72).

The reaction is therefore independent of the concentration of A. In some reactions can the concentration of A can be a limiting factor. In these type of reactions, the concentration of A eventually becomes so low that there is not sufficient amount of A to proceed with the reaction. These type of reactions is not strictly zero-order, but appears to be. Such a reaction is called a pseudo zero-order.

5.3 First-order reactions

Consider hypothetical reaction 5.10 (Ritchie, 1966: 6).

$$A \xrightarrow{k_1} B \qquad (5.10)$$

The rate of a first order reaction (equation 5.11) is dependant on the concentration of only one reactant. This means that the amount d[A], undergoes chemical change in the short time interval dt, and is dependant only on the amount of A present at that instant (assuming that there is no change in volume, temperature or any factor that may affect the reaction).

$$-d[A] / dt = k_1[A] \qquad (5.11)$$

After integration equation 5.11 can be written as follow (equation 5.12).

$$\ln[A] = \ln[A]_0 - kt \qquad (5.12)$$

Because $[A]_0$ is a constant, equation 5.12 can be put in the form of a straight line with y = ln[A], m = -k and b = $\ln[A]_0$. A graph of ln[A] versus time will be linear with a slope of –k (illustrated in figure 5.3.

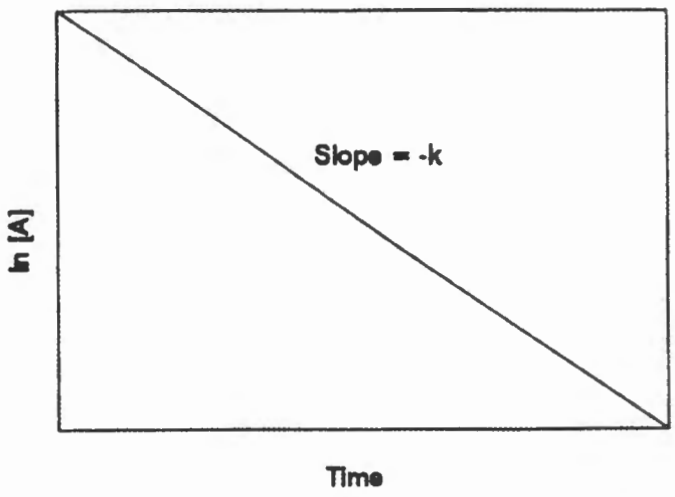


Figure 5.3: First-order plot for A → B (House, 1997: 5).

Equation 5.12 can also be written in the form (given in equation 5.13):

$$[A] = [A]_0 e^{-kt} \qquad (5.13)$$

Equation 5.12 can also be written (given in equation 5.14) to define $[A]_0$ (the initial amount of the reacting material) and x (the amount that reacts in time t), in order to calculate the amount of A remaining after time t (Capellos and Bielski, 1972: 8).

$$dx/\,dt = k_1([A]_0 - x) \qquad (5.14)$$

At time t = 0, $[A]_0$ is 0.
At time t = t, $([A]_0 - x)$ is x

The unit of the rate constant for a first-order reaction is a number per unit of time (usually sec^{-1}).

5.3.1 Half-life determination for a first-order process

The half-life of a zero-order reaction can be determined by equation 5.15 (House, 1997: 6).

$$k = 2.303/\,t_{\frac{1}{2}}\,(\log 1/\,2) \qquad (5.15)$$

$\therefore\ k = 0.693/\,t_{\frac{1}{2}}$

The units of $t_{\frac{1}{2}}$ depend on the units for k. If k is sec^{-1}, then $t_{\frac{1}{2}}$ is sec.

Half-life is easy to calculate when [A] is plotted versus time. Half-life is the time where [A] is equal to 0.5. A graphical presentation of [A] versus time is illustrated in figure 5.4.

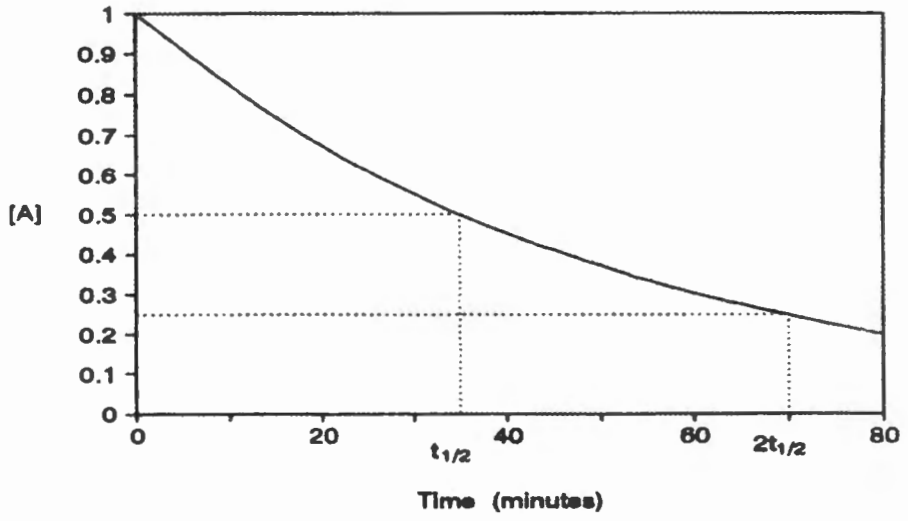


Figure 5.4: Half-life determination for a first-order process (House, 1997: 7).

5.3.2 Pseudo first-order reactions

Consider the following reaction (equation 5.16) (Logan, 1996: 8).

$$A + C \xrightarrow{k_1} Product$$
$$B + C \xrightarrow{k_2} Products \qquad (5.16)$$

This mechanism consists of two reactions in which A and B compete for C. Each reaction could be studied independently of the other. To find a solution for the two concurrent reactions, experimental conditions can be adjusted so that second-order rate constants are studied under pseudo first-order conditions. The rate of a pseudo first-order reaction can be given by equations 5.17 and 5.18 (Logan, 1996: 8).

$$-d[A]/dt = k_1[A][C] \qquad (5.17)$$
$$-d[B]/dt = k_2[B][C] \qquad (5.18)$$

Pseudo first-order conditions can be imposed by making the concentration of C much greater than A or B (Capellos and Bielski, 1972: 72).
The concentration of C remains approximately constant during the reaction and can be written as equations 5.19 and 5.20.

$$-d[A]/dt = k_1[C][A] \quad (5.19)$$

$$-d[B]/dt = k_2[C][B] \quad (5.20)$$

Integration yields equations 5.21 and 5.22.

$$[A] = [A]_0 e^{-k1[C]t} \quad (5.21)$$

$$[B] = [B]_0 e^{-k2[C]t} \quad (5.22)$$

The concentration of A and B which have reacted with C in time t is given by $[A]_0[1 - e^{-k1[C]t}]$ and $[B]_0[1 - e^{-k2[C]t}]$, respectively. The amount of C which has reacted in time t is equal to the sum of A and B which have reacted in the same time interval and can be given as equation 5.23.

$$[C]_0 - [C] = [A]_0[1 - e^{-k1[C]t}] + [B]_0[1 - e^{-k2[C]t}] \quad (5.23)$$

5.4 Second-order reactions

A second-order reaction usually results from a reaction given in equation 5.24 (Capellos and Bielski, 1972: 14).

$$A + B \xrightarrow{k_2} C \quad (5.24)$$

At time t = 0, amount of C is 0.
At time t = t, $A = ([A]_0 - x)$; $B = ([B]_0 - x)$; and $C = x$

If $[A]_0$ and $[B]_0$ represent the initial quantities of the two reacting chemicals A and B, and x is the number of moles that react in given time t. The rate at which C is formed can be written as equation 5.25.

$$dx/dt = k_2([A]_0 - x)([B]_0 - x) \quad (5.25)$$

If the initial concentration of A is equal to the initial concentration of B ($[A]_0 = [B]_0$), equation 5.24 can be written as equation 5.26:

$$2A \xrightarrow{k_2} C \quad (5.26)$$

With integration the second rate constant can be written as equation 5.27.

$$1/[A] - 1/[A]_0 = kt \quad (5.27)$$

Since the initial concentration of A is a constant, the equation can be put in the form y = mx + c (given in equation 5.28).

$$1/[A] = kt + 1/[A]_0 \quad (5.28)$$

A plot of 1/ [A] versus time (illustrated in figure 5.5) should be linear with a slope of k and an intercept of $1/[A]_0$

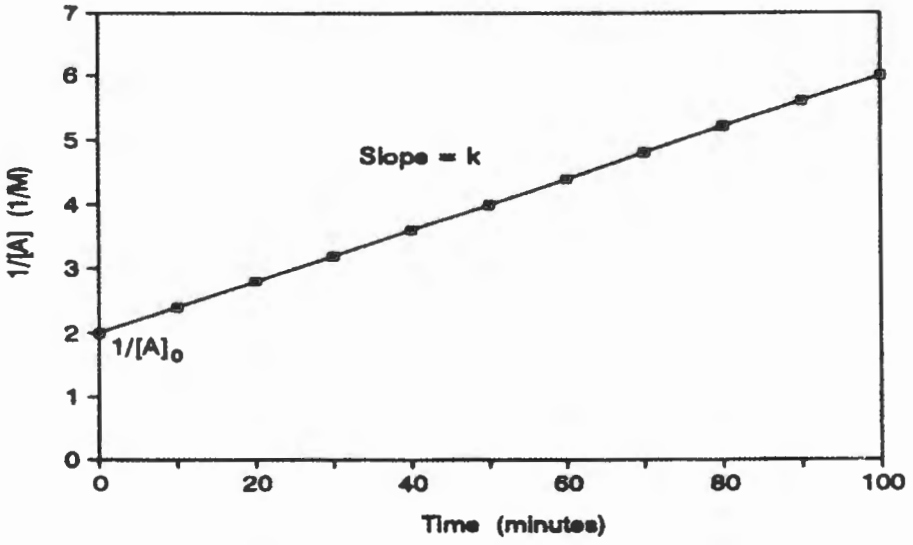


Figure 5.5: Second-order rate plot 2A → C (House, 1997: 8).

5.4.1 Half-life determination for a second-order process

The half-life for a second-order rate law can easily be calculated (House, 1997: 8). At time equal to one half-life, the concentration of A has decreased to one-half its original value and can be written as equation 5.29.

$$1/([A]_0/2) - 1/[A]_0 = kt_{\frac{1}{2}} \quad (5.29)$$

Equation 5.20 can also be written as equation 5.30.

$$t_{\frac{1}{2}} = 1/k[A]_0 \quad (5.30)$$

5.5 Third-order reactions

A chemical reaction is to be third-order if its rate depends on three concentration terms. Third-order reactions are very rare and will only be discussed very shortly.

Consider equation 5.31 (Capellos and Bielski, 1972: 14).

$$A + B + C \xrightarrow{k_3} Products \qquad (5.31)$$

If the concentrations of A, B and C are equal then (A, B and C = $[A]_0$):
At time t = 0, $[A]_0$, = 0
At time t= t, ($[A]_0 - x$) = x

The third-order rate can then be written as equation 5.32.

$$dx/ dt = k_1 ([A]_0 - x)^3 \qquad (5.32)$$

5.5.1 Half-life determination for a third-order process

The half-life for a third-order rate law can be calculated using equation 5.34 (House, 1997: 36).

$$t_2 = 3/ 2k_1[A]_0 \qquad (5.34)$$

5.6 Conclusion

Chemical kinetics is concerned with the analysis of chemical reactions. The raw data of chemical kinetics are measurements of rates of reactions. Chemical kinetics provide no information on the energy or stereochemical state of the individual molecules. However it does have the valuable potential to break down complex mechanisms into sequences of simple reactions.

Chapter 6: Experimental

6.1 Physical properties of PHEA-fenoprofen conjugate

6.1.1 Melting range

DSC thermograms of PHEA-fenoprofen conjugate were recorded with a Shimadzu DSC-50 instrument. The measurement conditions were as follows: sample weight: approximately 2 mg; sample holder: aluminium crimp cell; gas flow: nitrogen at 45 ml/min; heating rate: 10 °C per minute.

6.1.2 Solubility of PHEA-fenoprofen conjugate

Solubility of PHEA-fenoprofen conjugate was determined by preparing saturated solutions of PHEA-fenoprofen conjugate in different solvents and leaving them to rotate for 48 hours at room temperature (22 °C). Excess PHEA-fenoprofen conjugate was then filtered off and the amount conjugate in solution was determined by UV absorption for fenoprofen in the different solvents, using a Shimadzu UV 2100® spectrophotometer at 272 nm.

6.1.3 Ultraviolet absorption spectrum

The UV spectra of fenoprofen calcium, PHEA and PHEA-fenoprofen conjugate was recorded on a Shimadzu UV 2100® spectrophotometer at 272 nm.

6.1.4 X-ray powder diffractometry

Diffractometric profiles were obtained at room temperature with a Bruker D8 advance diffractometer (Bruker Germany). The measurements conditions were: target, Cu: K_β-filter, Ni: voltage, 40 kV, current, 30mA, divergence slit, 2 mm, anti scatter slit, 0.6 mm: detector slit, 0.2 mm scanning speed, 2 °/min (step size 0.025°, step time, 0.75 sec). Approximately 200mg sample was loaded into an aluminium sample holder, taking care not to introduce a preferential orientation of the crystals.

6.1.5 Infrared spectrum

The infrared spectrum of PHEA-fenoprofen conjugate was recorded on a Perkin-Elmer 457 spectrometer over a range of 500 – 4000 cm^{-1}.

6.2 HPLC method development

6.2.1 Introduction

In order to fully characterise the fenoprofen prodrug, it is necessary to quantitate the prodrug in different matrixes, depending on the purpose of the analysis. It is therefore essential to utilise a robust HPLC method that can selectively, accurately and precisely quantitate the prodrug in various matrixes with an acceptable limit of quantification.

This section deals with the experimental details necessary for the selection of a suitable chromatographic system for the quantification of the PHEA-fenoprofen conjugate. Chromatographic parameters such as capacity factor, peak symmetry, and theoretic plate count were used to evaluate the chromatography and column performance.
The most suitable chromatographic system was then subjected to extensive validation in order to establish acceptable performance criteria such as accuracy, precision, linearity, range and robustness for the analytical procedure.

6.2.2 Instrumentation

The HPLC instrumentation consisted of a spectraSYSTEM AS 3000 autosampler with a variable volume loop injector, a spectraSYSTEM P1000 isocratic pump and a spectraSYSTEM UV 1000 programmable variable wavelength detector with a 10 mm analytical flow cell. The analog detector signal is converted to a digital signal through a spectraSYSTEM SN 4000 signal converter. The converted signal is fed into a Pentium® MMX 166MHz computer where integration of the signal is accomplished by means of a TSP® PC 1000 software package with an IBM® OS/2 Warp version 3 operating system. The whole HPLC system is manufactured by THERMO SEPARATIONS® and supplied by SMM®[1].

1. SMM instruments: SMM House, 543 Kyalami Boulevard, Kyalami Park, Midrand 1685, South Africa.

6.2.3 Column selection

The selection of a suitable column was guided by the HPLC method described in the monograph for fenoprofen calcium in the USP (2000: 709). A LUNA 5 μ C_{18} and C_8 column by PHENOMENEX® as supplied by SEPARATIONS[2] was utilised.

Although the USP method utilises a C_{18} column, a C_8 column was also included for comparative and optimising purposes. The LUNA brand columns were selected for employing the latest advances in silica and bonding. The silica used is ultra pure and has remarkable surface smoothness, improving stability and column efficiency (Phenomenex, 1999: 177). The column is also known for its pH for improved resolution, separation and ruggedness of acids and bases. The particulars of the columns used are given in table 6.1.

Table 6.1: Particulars of the LUNA columns used.

	Unit	LUNA 5 μ C_{18}	LUNA 5 μ C_8
Dimensions	mm	150 x 4.60	150 x 4.60
Part No.		OOG - 4252 - EO	OOF - 4249 - EO
Serial No.		320775	336423
Batch No.		5291 - 14	5275 - 9
Particle size	μm	4.90	4.90
Particle distribution	90% /10%	1.96	1.96
Pore diameter	$^{o}_{A}$	100	100
Surface Area	m^2/g	405	405
Total carbon content	%	17.89	14.26
Surface coverage	μmol/ m^2	3.44	4.65

2. Separations: P.O Box 4181, Randburg, South Africa. (sepasales@iafrica.com)

6.2.4 Preparation of standards

Molecular mass of fenoprofen = 242.3. Molecular mass of fenoprofen calcium = 558.60. Purity of fenoprofen calcium raw material[3]: 101.6%. Dilutions were calculated as given in table 6.2.

Table 6.2: Calculated concentrations for fenoprofen calcium and fenoprofen.

Mother solution: 50 mg fenoprofen calcium → 50 ml		1000 μg/ml fenoprofen calcium 434 μg/ml fenoprofen
Amount taken from mother solution and made up to volume	Concentration fenoprofen calcium (μg/ml)	Concentration fenoprofen (μg/ml)
3 ml → 20 ml	150	66.10
3 ml → 25 ml	120	52.1
2 ml → 20 ml	100	43.4
2 ml → 25 ml	80	34.7
1 ml → 15 ml	66.6	28.9

6.3 Experimental procedure

The USP (2000: 709) HPLC method for fenoprofen calcium was used. To optimise peak symmetry the composition and flow rate of the mobile phase were slightly altered. The operating conditions are given in table 6.3.

Table 6.3: Adapted analytical conditions.

Temperature:	Ambient (approximately 25 ∀ 2 ^{0}C)
Mobile phase flow rate:	1.75 ml/ min
Detector:	Ultraviolet 272 nm
Sample size:	20 μ l
Mobile phase:	Acetonitrile: 60 volumes Concentrated phosphoric acid: to pH 2 Water up to 100 volumes

3. Fenoprofen calcium supplied by Eli Lilly South Africa: 50 Commando Road, Industria West Batch No: 502DH8

A comprehensive method validation was done on the C_8 column because the USP method was changed outside its original scope and also to prove that this method is suitable for its intended purpose (to analyse the conjugate in various matrixes in order to establish selected properties of this new prodrug).

6.4 Method validation for fenoprofen using a C_8 column

6.4.1 Specificity

To prove the specificity of the method for fenoprofen, five different concentrations of fenoprofen were spiked with a 1000 μg/ml solution of PHEA (the carrier used in the synthesis of the fenoprofen prodrug). A very high concentration PHEA was used to prove beyond doubt that PHEA has got no effect on the fenoprofen peak. PHEA was used because it is the only possible interfering substance present (other than fenoprofen) in the sample. It is therefore very important that the PHEA peak (if any) does not interfere. Interference from any degradation products was not anticipated, as both PHEA and fenoprofen are stable under the experimental conditions.

6.4.2 Linearity

Linearity was determined by a series of five standard concentrations whose concentrations ranged between 80% - 120% of the expected concentration range, as calculated in paragraph 1.4.1. Linearity was also evaluated graphically by visual inspection of a plot of peak area versus the concentration (μg/ml) of the fenoprofen standard. A non-zero intercept demonstrates that there is no effect on the accuracy of the method (Brittain 1997: 277). Linearity was also evaluated by means of a linear regression analysis of the standard curve.

6.4.3 Precision

Precision was determined by taking six aliquots of a homogenous sample under standard conditions required for the performance of this method. These measurements were used to calculate the relative standard deviation.

6.4.3.1 Intermediate precision

Intermediate precision was determined by using the same analytical method and conditions, but on different instruments on different days. The results obtained from primary validation were compared with the data generated during the secondary validation.

6.4.4 Robustness

Robustness was tested by deliberately changing the pH of the mobile phase from 2.0, (USP, 2000: 709), to 1.8 and 2.2 (pH levels lower than 1.8 and higher than 2.2 caused peak distortion and produced unacceptable chromatograms). Five different target concentrations were used, and injected at different mobile phase pH levels, using the same method and conditions. The percentage recovery, average recovery, standard deviation and percentage relative standard deviation were calculated.

6.4.5 Accuracy

The method's accuracy was determined by applying the procedure to samples spiked with known amounts of fenoprofen spanning concentration levels of 66, 80, 100, 120 and 150 %. Accuracy is expressed as the percentage recovery of fenoprofen that was retrieved after 6 injections of each concentration.

6.4.6 Limit of detection (LOD) and quantitation (LOQ)

The limit of detection and of quantitation was determined by analysing a number of low concentration samples. The concentration of these samples spanned the lowest quarter of the range established during the linearity study. Each sample was assayed six times, and the standard deviation of the peak area calculated for fenoprofen. The standard deviation values were then averaged to find the mean standard deviation associated with this method for fenoprofen. This mean standard deviation is divided by the slope of the standard curve, to yield an estimate factor for the noise associated with this method. This noise factor is then multiplied by a factor three to calculate LOD. To calculate LOQ, the calculated noise factor is multiplied by ten.

6.5 Fenoprofen release from PHEA

6.5.1 Determination of PHEA-fenoprofen conjugate drug loading

The percentage of fenoprofen present in PHEA-fenoprofen conjugate ester was determined by the hydrolysis method described by Giammona *et al.*, (1994: 59). 0.075g of PHEA-fenoprofen conjugate was weighed and dissolved in 50 ml of boric acid buffer solution. The boric acid buffer consisted of 3.1 g boric acid, 3.7 g potassium chloride and 0.2 M sodium hydroxide (to adjust pH to 10) and made up with distilled water to 1000 ml. This reaction mixture was maintained at 60 ± 0.1 °C for 72 hours. Each sample was neutralised with 2 N hydrochloric acid and analysed by HPLC. With every time interval samples were taken and each sample was injected 5 times. The experiment was repeated 6 times.

A fenoprofen calcium sample (equivalent to 52.1 μg/ml of free fenoprofen) dissolved in boric acid buffer was also kept at 60 °C for 72 hours to ensure that fenoprofen itself showed no breakdown under these conditions. A fenoprofen sample was injected three times (after 24 hours, 48 hours and 72 hours) during the 72 hour period.

6.5.1.1 Spectrophotometry

Fenoprofen loading was confirmed by using UV spectrophotometry. Two solutions were prepared. The first contained 43.4 μg/ml of fenoprofen and the second contained PHEA-fenoprofen conjugate equivalent to 43.4 μg/ml of fenoprofen (0.00618 mg PHEA-fenoprofen conjugate). The maximum absorption of fenoprofen in both solutions was measured at 272 nm.

6.5.1.2 Fenoprofen release from PHEA-fenoprofen conjugate (pH 10, 37 °C).

The same method that was used to determine drug loading was also used to determine fenoprofen release from PHEA at 37°C at pH 10. The same amounts of samples were again analysed, using the HPLC method for fenoprofen described in paragraph 6.4. The same standard curve for fenoprofen was also used (given in appendix 1). A fenoprofen calcium sample (equivalent to 52.1 μg/ml of free fenoprofen) and dissolved in boric acid buffer was also kept at 37 °C for 300 hours to ensure that fenoprofen itself showed no breakdown under these conditions. A fenoprofen sample was injected three times (after 24 hours, 72 hours, 100 hours and 300 hours) during the 300 hour period.

6.5.1.3 Fenoprofen release from PHEA-fenoprofen conjugate (pH 1.1, 37 °C).

0.075g of PHEA-fenoprofen conjugate was weighed and dissolved in 50 ml of glycine buffer solution and 5 ml dimethylsulphoxide as co-solvent. As dimethylsulphoxide caused UV interference resulting in a reduction in peak area, a new standard curve for fenoprofen with dimethylsulphoxide was used to compensate for UV disturbances.
The glycine acid buffer consisted of 6.0 g of glycine, 4.6 g of sodium chloride, 1.0 M hydrochloric acid (to adjust pH to 1.1) and made up with distilled water to 1000 ml. This reaction mixture was maintained at 37 ± 0.1 °C for 800 hours. Each sample was neutralised with 2 N sodium hydroxide and analysed by HPLC (method described in chapter 6). With every time interval samples were taken and each sample was injected 5 times. The experiment was repeated 6 times. The standard curve used to calculate the obtained fenoprofen concentrations, the values obtained during hydrolysis and a representative chromatogram of fenoprofen released during hydrolysis are given in appendix 3. A fenoprofen calcium sample (equivalent to 52.1 μg/ml of free fenoprofen) and dissolved in glycine buffer was also kept at 37 °C for 800 hours to ensure that fenoprofen itself showed no breakdown under these conditions. A fenoprofen sample was injected three times (after 24 hours, 400 hours and 800 hours) during the 800 hour period.

6.6 PHEA-fenoprofen conjugate powder dissolutions

6.6.1 Dissolution in simulated gastric juice (pH 1.2) at 37 °C

Six samples of 0.100 g of PHEA-fenoprofen conjugate were weighed and each sample vortexed in 10 ml of simulated gastric juice for 2 minutes. Dissolution conditions that were used are given in table 6.4. Each sample was analysed using HPLC.

Table 6.4: Dissolution conditions used with simulated gastric juice

Dissolution medium	Simulated gastric juice (USP2000: 2235): 2 g sodium chloride, 3.2 g purified pepsin[4], 0.2 M hydrochloric acid (to adjust pH to 1.2), water to 1000 ml.
Volume of dissolution medium	900 ml
R.P.M	100
Apparatus [5]	Paddles
Sampling times	5, 10, 15, 30, 45, 90, 120, 200, 500, 700 minutes

6.6.2 Fenoprofen release from PHEA in simulated intestinal fluid

Six samples of 0.100 g of PHEA-fenoprofen conjugate were weighed and each sample vortexed in 10 ml simulated intestinal fluid for 2 minutes. . Dissolution conditions that were used are given in table 6.5.

Table 6.5: Dissolution conditions used for simulated intestinal fluid

Dissolution medium	Simulated intestinal fluid (USP, 2000: 2236): 6.8 g monobasic potassium phosphate, 10 g pancreatin[6], 0.2 M sodium hydroxide (to adjust pH to 6.8), water to 1000 ml.
Volume of dissolution medium	900 ml
R.P.M	100
Apparatus	Paddles
Sampling times	5, 10, 15, 30, 45, 90, 120, 200, 500, 700 minutes

4. Purified pepsin (with activity 800 – 2500 units/ mg protein) supplied by Merck (Midrand South Africa), batch number 1010486.

5. Vankel VK 7000® supplied by Vankel Industries Inc. 36 Meridian road, Edison, NJ 08820 USA.

6. Pancreatin from porcine pancreas supplied by Merck (Midrand, South Africa), batch number 232-68-9

Chapter 7: Results and discussion

7.1 Physical properties of PHEA-fenoprofen conjugate

7.1.1 Melting range

PHEA-fenoprofen conjugate melts at 269.71 °C (illustrated in figure 7.1), in comparison with fenoprofen, with melting point at 118 °C – 123 °C. Because no peak is visible near 118 °C – 123 °C, it means that fenoprofen is chemically bound to the PHEA carrier (not just merely dispersed or incorporated into PHEA) and that no free fenoprofen is present in the PHEA-fenoprofen conjugate.

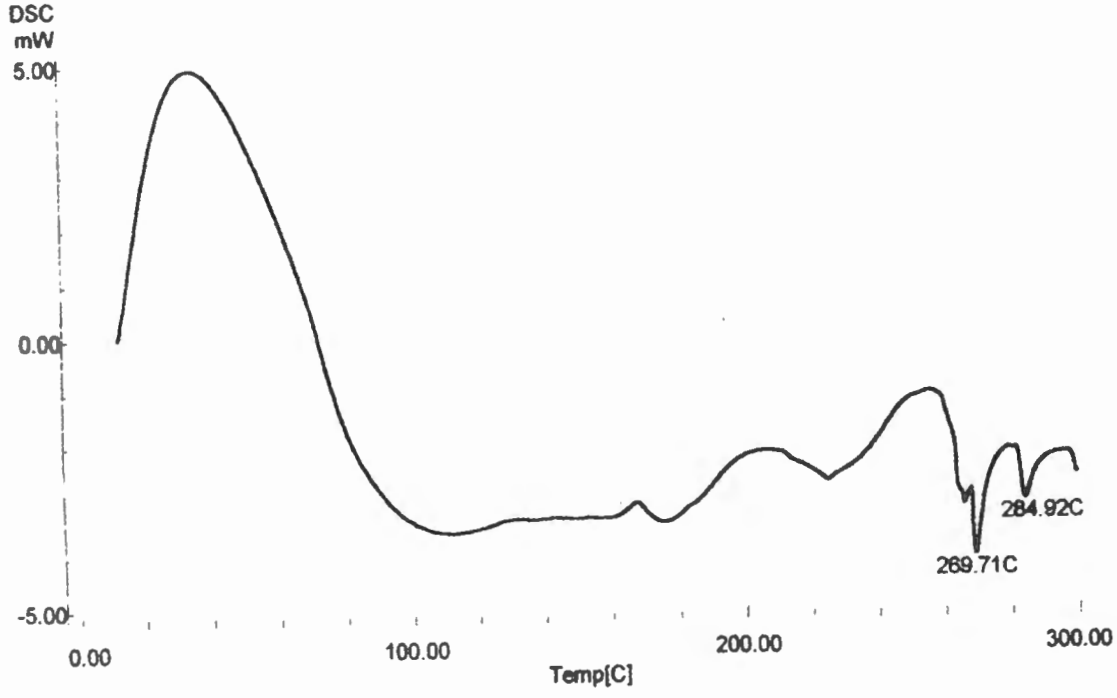


Figure 7.1: DSC-thermogram of PHEA-fenoprofen conjugate (rate: 10 °C/ min).

7.1.2 Solubility

The solubility of PHEA-fenoprofen conjugate in different solvents are given in table 7.1.

Table 7.1: Solubility of PHEA-fenoprofen conjugate in different solvents

Solvent	Solubility (mg/ml)
Ethanol	Insoluble
Methanol	Insoluble
Acetonitrile	Insoluble
THF	Insoluble
Cyclohexane	Insoluble
Diethylether	Insoluble
Chloroform	Insoluble
Water	89.21 (concentration level where PHEA-fenoprofen conjugate turns into a gel)

PHEA-fenoprofen conjugate is totally insoluble in all organic solvents tested. In water, dimethylsulphoxide (DMSO) and dimethylformamide (DMF), the PHEA-fenoprofen conjugate turns into a gel at high concentrations, making it difficult to determine the saturation level.

7.1.3 Ultraviolet absorption spectrum

Because PHEA shows no UV absorption between 250 – 300 nm (given in figure 7.2), the amount fenoprofen can be determined without any interference using spectrophotometry. Because PHEA-fenoprofen conjugate is insoluble in methanol, the UV spectrum was drawn (given in figure 7.3) using 50:50 methanol:water. The spectrum exhibits a maximum absorption peak at 274 nm, in comparison to fenoprofen with maximum absorption at 272 nm.

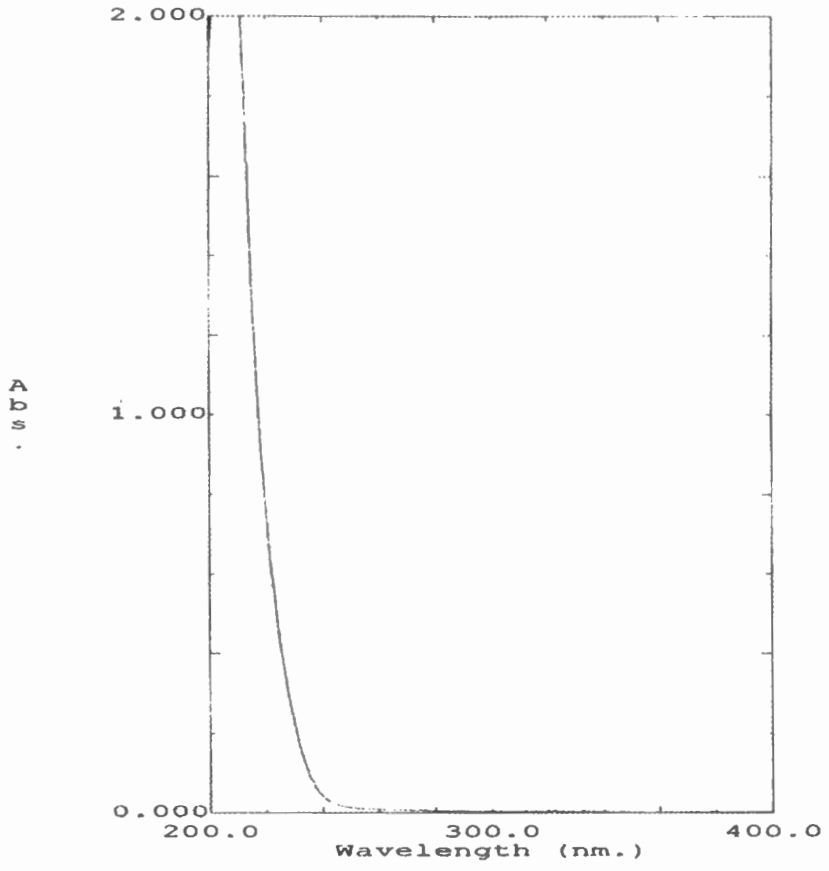


Figure 7.2: The ultraviolet spectrum of PHEA

This method of analysis only gives the amount fenoprofen present, without any differentiation between fenoprofen bound to PHEA and free fenoprofen (released from PHEA). Spectrophotometry can thus not be used where fenoprofen release from PHEA is studied. This method can also not be used in dissolution studies, because the amount of free fenoprofen can not be determined. To determine PHEA-fenoprofen conjugate drug loading, spectrophotometry can be used successfully.

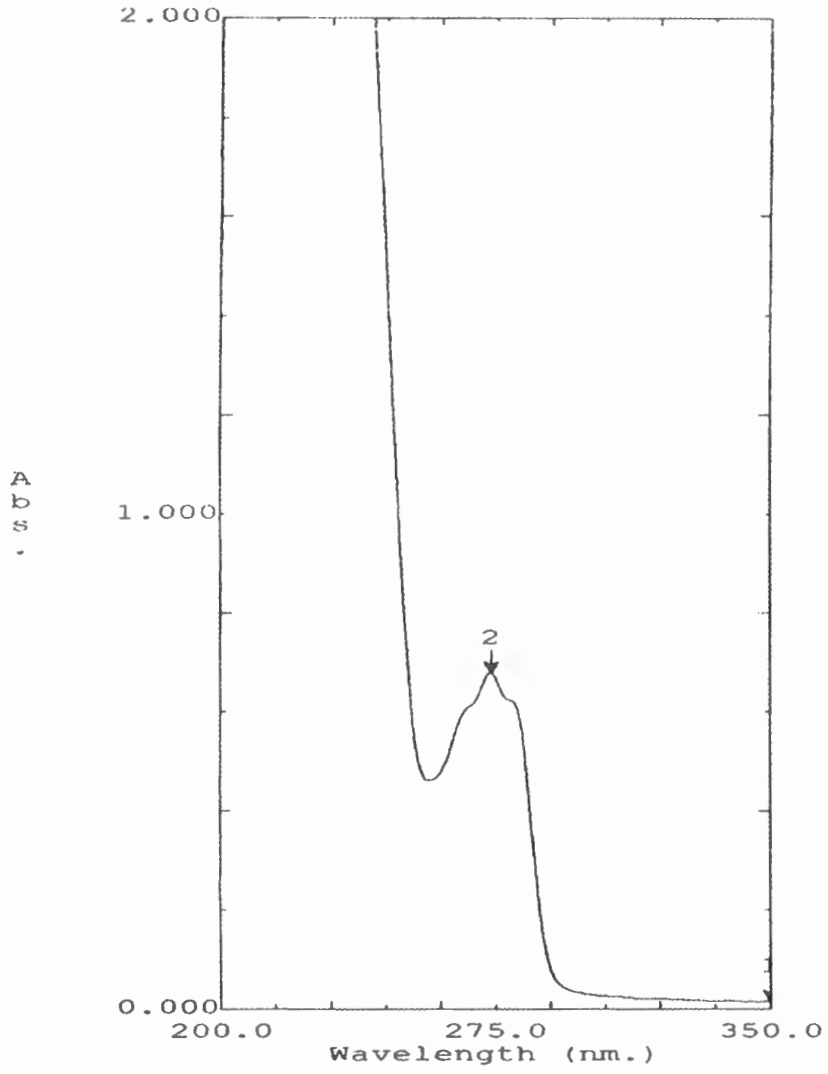


Figure 7.3: The ultraviolet spectrum of PHEA-fenoprofen conjugate

7.1.4 X-ray powder diffractometry

The PHEA-fenoprofen conjugate shows a totally new x-ray powder diffractometric pattern (figure 7.5), in comparison to fenoprofen calcium (figure 7.4). It proves that there is no free fenoprofen present in the PHEA-fenoprofen conjugate and that fenoprofen is chemically bound to PHEA, giving rise to a new molecule with new physical properties.

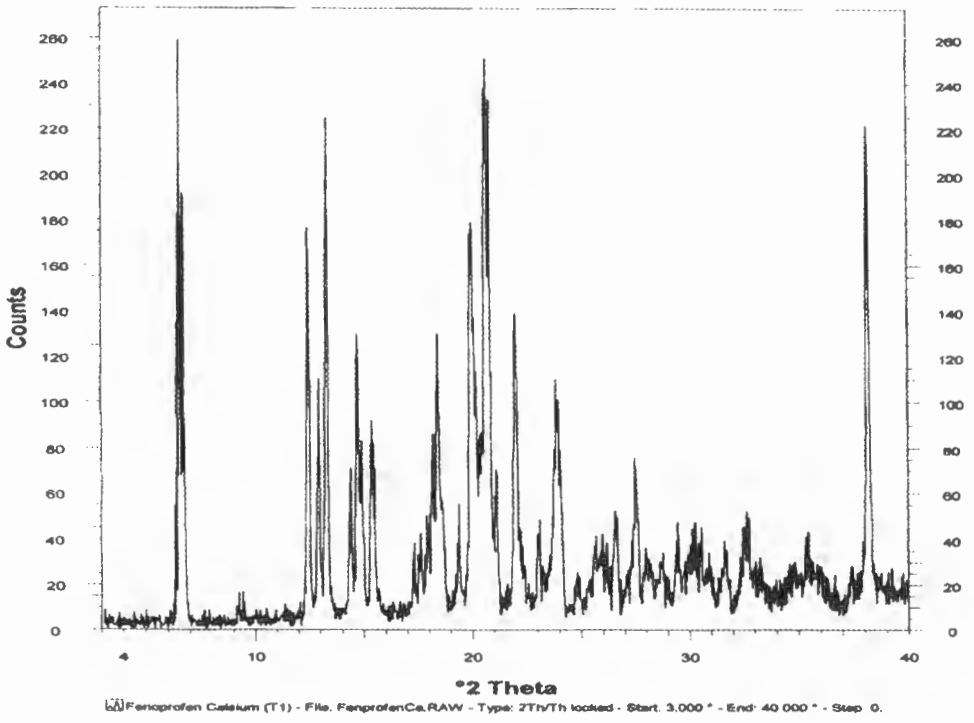


Figure 7.4: X-ray powder diffractometric pattern of fenoprofen calcium.

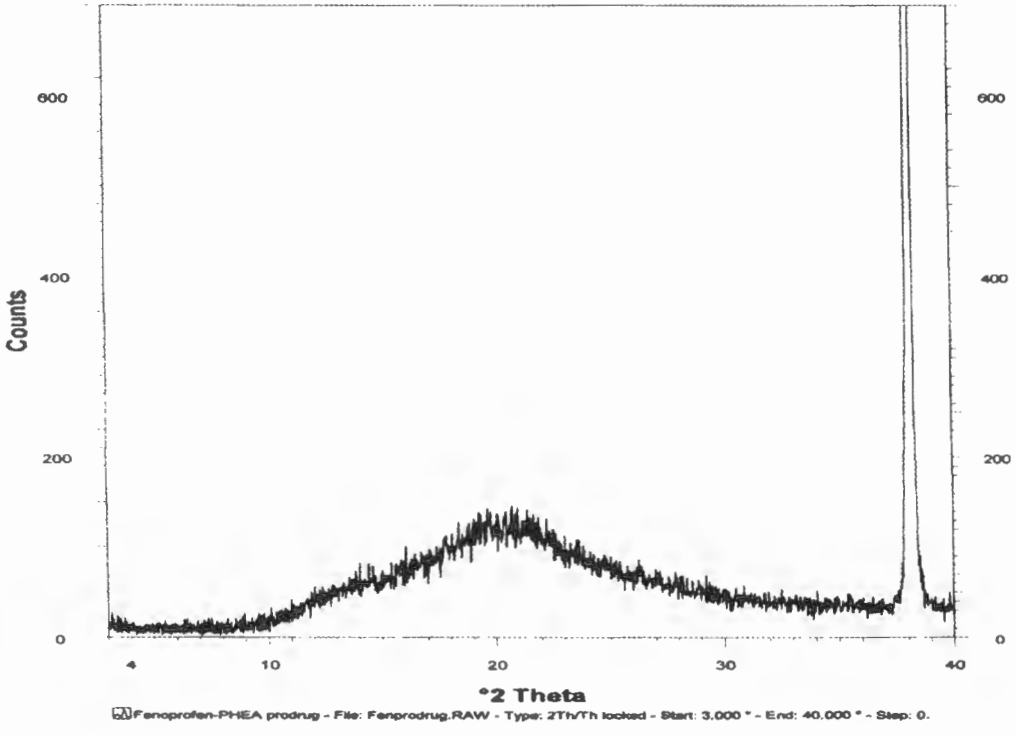


Figure 7.5: X-ray powder diffractometric pattern of PHEA-fenoprofen conjugate.

7.1.5 Infrared spectrum

The infrared spectrum of PHEA-fenoprofen conjugate is given in figure 7.6.

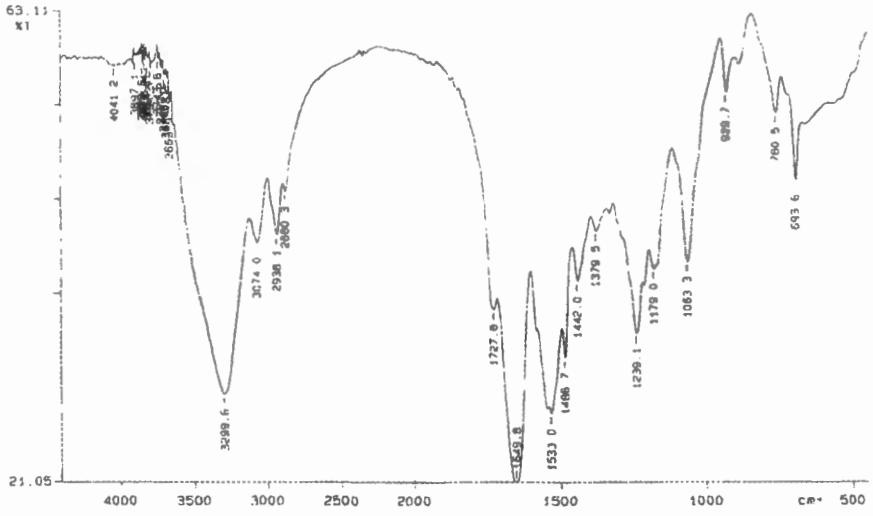
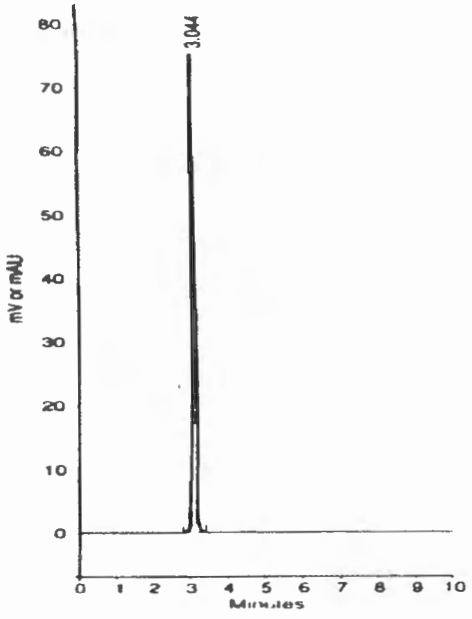


Figure 7.6: Infrared spectrum of PHEA-fenoprofen conjugate.

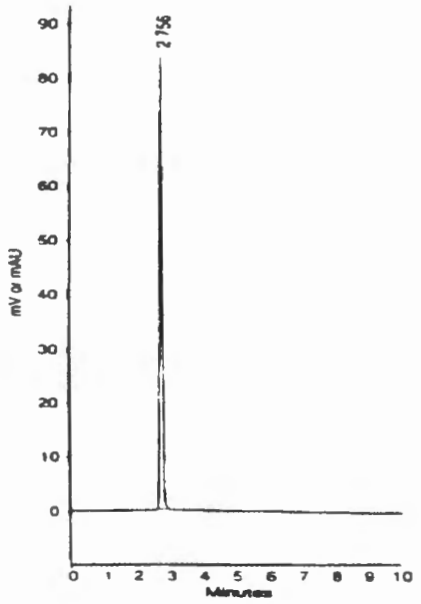
7.2 HPLC method development

Both the C_{18} and C_8 columns were evaluated using the same instrument and analytical conditions. Representative chromatograms of fenoprofen on both C_{18} and C_8 columns are given in figure 7.7.



(1)

Figure 7.7: Representative chromatograms of fenoprofen calcium on C_{18} (1) and C_8 (2) columns.



(2)

Figure 7.7 (continued): Representative chromatograms of fenoprofen calcium on C_{18} (1) and C_8 (2) columns.

The performance data for fenoprofen on both the C_{18} and C_8 columns were compared. These data are given in table 7.2.

Table 7.2: Performance data comparison between C_{18} and C_8 columns.

Performance parameters	C_{18}	C_8
Capacity factor	10.20	8.70
Retention time (min)	3.20	2.80
Peak symmetry factor	1.42	1.20
Plates/meter	50800	52580
HETP (mm)	0.02	0.01

From the comparative data, it can be seen that the performance of the C_{18} column is not a significantly different from that of the C_8 column. The C_8 column was selected because of better peak symmetry and shorter retention time in an effort to optimise the fenoprofen method.

7.3 A comprehensive method validation for fenoprofen using a C_8 column

7.3.1 Specificity

A representative chromatogram of the spiked fenoprofen solution is shown in figure 7.8.

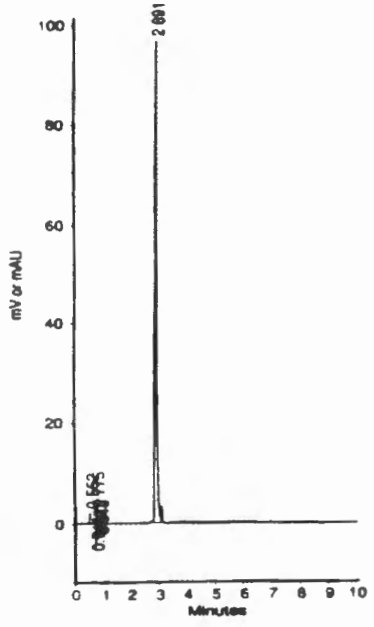


Figure 7.8: A representative chromatogram of the PHEA spiked fenoprofen solution.

To prove the specificity of the fenoprofen HPLC method mathematically, five different concentrations of fenoprofen samples not containing PHEA, and five samples of fenoprofen spiked with PHEA, were analysed using the exact specified experimental procedure. The five concentrations used were chosen to span the range specified by the analytical procedure (as discussed earlier).

The bias between the pure fenoprofen and the spiked fenoprofen samples was determined by calculating the percentage agreement between the two using the equation below.

% Agreement = $(T_P / T_A) \times 100$

where:

T_P = test result in the presence of spiked fenoprofen

T_A = test result in pure fenoprofen samples

Specificity data and percentage agreements are given in table 7.3.

Table 7.3: Percentage agreement specificity data.

Sample number	Concentration fenoprofen found with PHEA present	Concentration fenoprofen found without PHEA present	Percent agreement
1	64.91	64.50	100.63
2	52.80	52.37	100.82
3	43.41	42.91	101.17
4	34.42	34.83	98.82
5	28.91	28.07	102.99
Average agreement (%)			100.886
Standard deviation			1.487
Relative standard deviation			1.474

A mean agreement of between 98 - 102% indicates the absence or any PHEA interference. In pharmaceutical specificity validation a % RSD of less than 2 % is acceptable (Huber, 1998: 8). The method is therefor regarded as specific for fenoprofen in the presence of PHEA.

7.3.2 Linearity

A graphic representation of the linearity data in the range 80 – 120 % is given in figure 7.9. The parameters generated by the linear regression analysis are given in table 7.4.

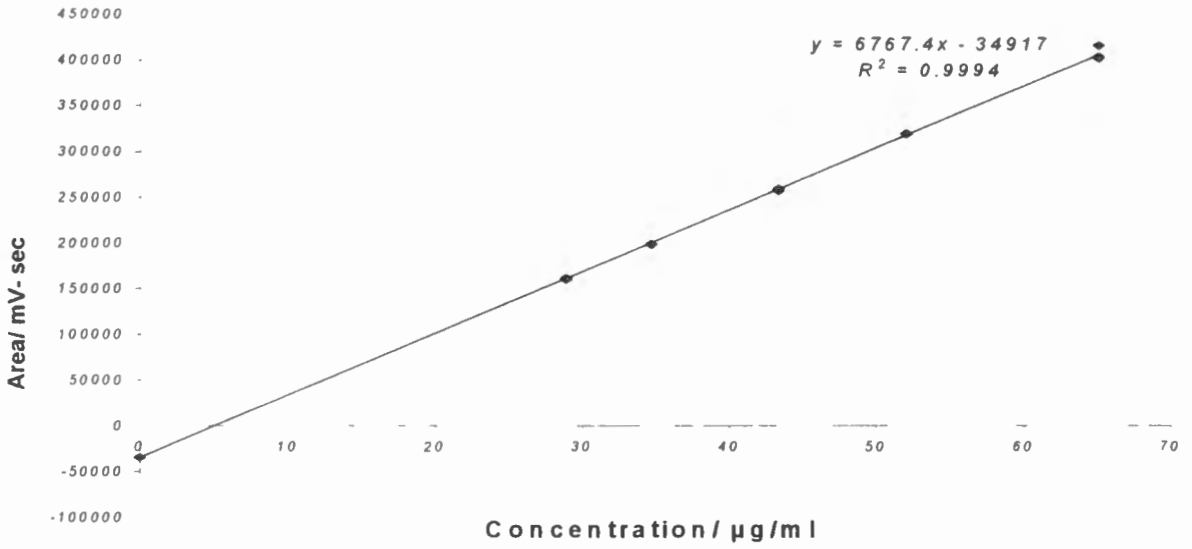


Figure 7.9: Graphical presentation of linearity plot of fenoprofen samples using a C_8 column.
Table 7.4: Linear regression parameters.

Regression statistics	
Multiple R	0.999
R Square	0.999
Adjusted R Square	0.998
Standard Error	3062.38
Observations	19

	Coefficients	Standard Error	t Stat	P value	Lower 95 %	Upper 95 %
Intercept	-34916.99	2406.25	-14.51	5.23E-11	-39993.75	-29840.2
X Variable 1	6767.42	50.80	133.21	4.15E-27	6660.24	6874.60

In the case of fenoprofen the correlation coefficient is 0.994 for the C_8 column, which is sufficient to estimate that the method is linear (CDER, 1994: 12, ICH, 1996: 4 and Brittain, 1997: 278).

7.3.3 Precision

Precision data are given in table 7.5.

Table 7.5: Precision data set after 6 measurements.

Sample	(N)	Instrument response (Area counts)	% RSD
1	5	265167	0.34
2	5	270493	0.22
3	5	271078	0.15
4	5	272183	0.40
5	5	271570	0.20
6	5	270506	0.31
Average		270166	
Standard Deviation		2533	
Relative Standard Deviation		0.94	

For raw material analysis in pharmaceutical quality control precision below 2 % is acceptable, while % RSD of less than 1 % should be easy to achieve (Brittain, 1997: 276).

7.3.3.1 Intermediate precision

The comparative data obtained the primary and secondary validation is given in table 7.6.

Table 7.6: Intermediate precision data set comparison for a C_8 column.

Analytical performance parameter	Value obtained by first HPLC instrument (H1)	Value obtained by second HPLC instrument (H2)	% Agreement
Precision (% RSD of the method)	0.94	0.96	102.13
Accuracy (percent recovery)	99.16	99.97	100.82
Linearity (correlation coefficient of linear regression analysis)	0.99	0.99	100.00

No hard and fast criteria exist for proof of intermediate precision, but the % agreement values for each parameter (illustrated in table 7.5), are all between 98 – 102 % and would be indicative of a precise method.

7.3.4 Robustness

Robustness data obtained are illustrated in table 7.7.

Table 7.7: Robustness validation comparison between pH 1.8, 2.0 and 2.2.

Target concentration (µg/ml) - pH 1.8, 2.0 and 2.2	Found concentration (µg/ml) - pH 1.8(µg/ml)	Found concentration (µg/ml) - pH 2.0	Found concentration (µg/ml) pH 2.2(µg/ml)	% Recovery		
				pH 1.8	pH 2.0	pH 2.2
28.93	28.07	28.97	28.83	97.13	100.24	99.76
34.72	34.83	34.54	34.90	100.37	99.54	100.58
43.40	44.70	43.40	43.30	100.99	100.00	99.77
52.08	52.33	52.32	52.76	100.44	100.42	101.27
65.10	64.51	65.16	64.96	99.09	100.09	99.78
Average recovery (%)				99.58	100.06	100.23
Standard deviation				1.55	0.33	0.67
% RSD				1.55	0.33	0.67

The linearity parameters of pH 2.2, 2.0 and 1.8 are illustrated in table 7.8.

Table 7.8: Linearity parameters of pH 2.2, 2.0 and 1.8.

Concentration (µg/ml)	% Agreement pH 1.8	% Agreement pH 2.0	% Agreement pH 2.2
65.1	99.68	100.08	100.33
52.1	100.50	99.52	99.08
43.4	100.22	99.96	100.06
34.7	100.34	99.48	100.25
28.9	97.01	100.10	99.66
% RSD	1.46	0.30	0.51
Average recovery	99.55	99.77	99.88
Standard deviation	1.45	0.30	0.51
Correlation coefficient	0.99	0.99	0.99

7.3.5 Accuracy

Accuracy data are given in table 7.9.

Table 7.9: Accuracy data set

Target concentration (%)	Spiked concentration (μg/ ml)	Found concentration (μg/ ml)	Percentage recovery
66	28.93	28.07	97.02
80	34.72	34.83	100.31
100	43.40	42.91	98.87
120	52.08	52.37	100.55
150	65.10	64.50	99.07
Average recovery			99.16
Standard deviation			1.41
Relative Standard Deviation (% RSD)			1.41

A % RSD of less than 2 % is acceptable to prove that a method is accurate. This method (with a % RSD of 1.41) is therefore regarded as accurate for fenoprofen as given by the USP (2000: 2150).

7.3.6 Limit of detection (LOD) and limit of quantitation (LOQ)

LOQ and LOD data set for fenoprofen is given in table 7.10.

Table 7.10: LOQ and LOD data set

	Instrument response (0.08 μg/ml)	Instrument response (0.17 μg/ml)	Instrument response (0.26 μg/ml)	Instrument response (0.34 μg/ml)
1	1084	1380	1660	2507
2	1166	1129	1739	2220
3	1199	1424	1945	2194
4	1184	1459	1737	2156
5	1132	1431	1789	2228
6	1222	1543	1779	2199

Table 7.10 (continued): LOQ and LOD data set

	Instrument response (0.08 μg/ml)	Instrument response (0.17 μg/ml)	Instrument response (0.26 μg/ml)	Instrument response (0.34 μg/ml)
Mean value	1164	1394	1774	2250
Standard deviation	49.83	140.79	94.94	128.06
Average standard deviation			103.40	
Slope of calibration curve			10356	
Limit of detection (LOD) (μg/ml)			0.02	
Limit of quantitation (LOQ) (μg/ml)			0.09	

These LOQ and LOD values will be very important if future *in vivo* studies need to be conducted using this method of analysis with biological samples for the PHEA-fenoprofen conjugate.

7.4 Conclusion

The correct performances of a well designed series of validation experiments were done to assure that all data obtained through the use of the analytical method are reliable. Even under deliberate stress conditions, like spiked contaminants, mobile phase pH changes, different instruments and time intervals, the method still proved to be highly precise, accurate, specific and robust.

For the pharmaceutical industry proper validation is very important. No regulatory committee will accept any data obtained by a non-validated method. This is why the validation of this method for fenoprofen is very important for this study. Proper validation will ensure reliable results generated by this study. It was also important that validation was done at the beginning of this study, not only to investigate possible shortcomings of this method, but also to assure that data generated in this study will be valid, making the whole study reliable and worthwhile.

7.5 Fenoprofen release from PHEA

7.5.1 Introduction

In order to determine the amount fenoprofen present in the PHEA-fenoprofen conjugate, spectrophotometric methods can be used (as described in paragraph 5.1.4). If drug release studies are to be conducted HPLC proved to be more effective because this method can differentiate between fenoprofen bound to PHEA and fenoprofen released from PHEA. HPLC can thus be used to study fenoprofen release from PHEA under different conditions and time intervals. As with the spectrophotometric method for PHEA-fenoprofen conjugate, PHEA exhibits no peak in HPLC (illustrated in figure 7.10).

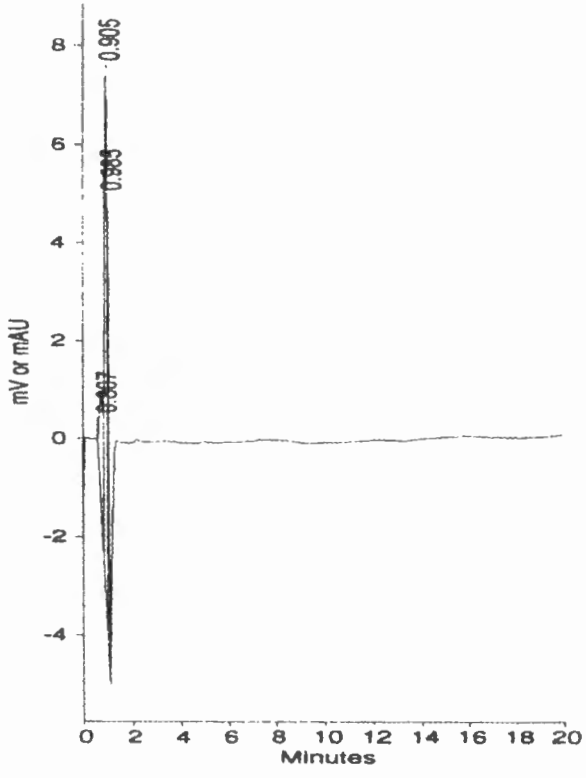


Figure 7.10: HPLC chromatogram of PHEA

No fenoprofen peak is also visible on a HPLC chromatogram of the PHEA-fenoprofen conjugate (illustrated in figure 7.11). This phenomenon is one method that can be used to prove that fenoprofen is chemically bound to PHEA (giving rise to a new molecule) and not merely dispersed or incorporated into PHEA. It can also prove that no free fenoprofen drug is present after PHEA-fenoprofen conjugate synthesis.

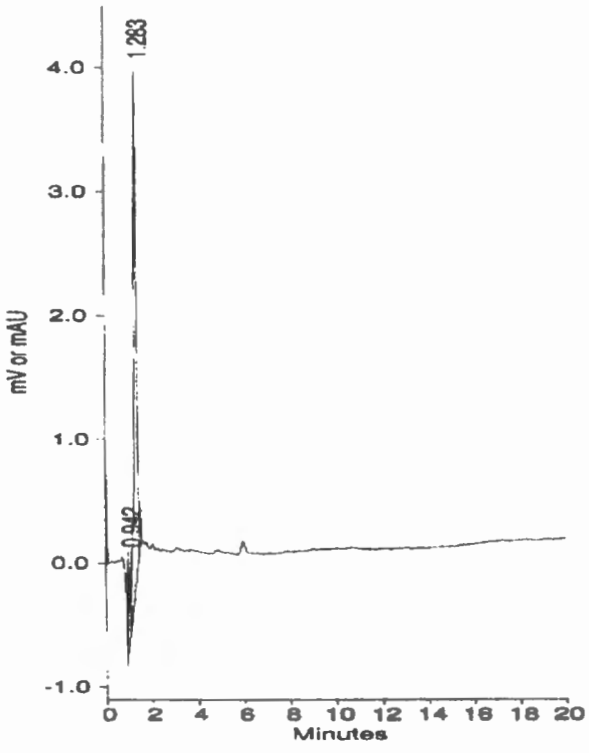


Figure 7.11: HPLC chromatogram of PHEA-fenoprofen conjugate.

Because fenoprofen concentrations cannot be determined by merely injecting a PHEA-fenoprofen conjugate sample on HPLC, it is necessary to chemically break the ester bond between fenoprofen and PHEA. Studies conducted by Giammona et al., (1994: 58) on diflunisal-PHEA drug ester and naproxen-PHEA drug ester (Giammona et al., 1989: 58), hydrolysis studies were used in order to release diflunisal and naproxen from PHEA. Not only can a wide variety of hydrolysis studies (in different mediums) be used, but conditions like temperature can be manipulated in order to accelerate or slow down the reaction.

7.5.2 Determination of PHEA-fenoprofen conjugate drug loading

Fenoprofen release from PHEA (60 °C, pH 10) is illustrated in figure 7.12.

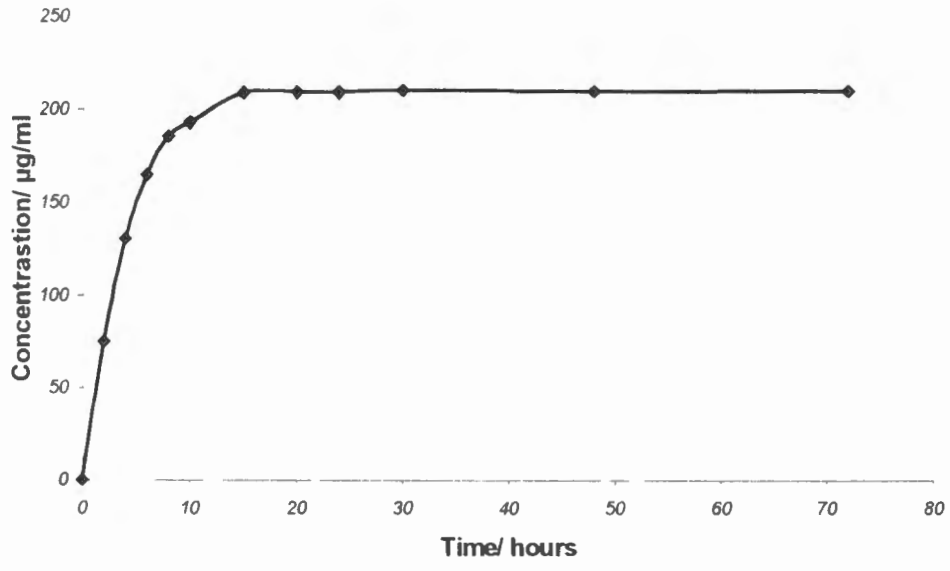


Figure 7.12: A graphical presentation of the concentration fenoprofen released over time to determine drug loading at 60 °C in boric acid buffer

Drug loading was calculated as follows: 75 mg of PHEA-fenoprofen conjugate was dissolved in 50 ml boric acid buffer (1500 µg/ml solution). The maximum average fenoprofen concentration detected by HPLC was 210.13 µg/ml. The amount of fenoprofen present in a 210.13 µg/ml solution of PHEA-fenoprofen conjugate was calculated to be 10.50 mg, which means there is 10.50 mg of fenoprofen present in every 75 mg of PHEA-fenoprofen conjugate. The ratio PHEA-fenoprofen conjugate: fenoprofen free drug was calculated to be 1:4.7. The amount fenoprofen free drug present can also be expressed as percentage drug loading (the amount fenoprofen in 100 g fenoprofen-PHEA). Percentage drug loading was calculated to be 14%. The fenoprofen calcium control sample (discussed in paragraph 6.5.1) showed no degradation throughout the experiment.

7.5.2.1 Determination of fenoprofen drug loading using spectrophotometry

Fenoprofen drug loading was confirmed by using UV spectrophotometry. Two solutions were made. The first contained 43.40 µg/ml fenoprofen and the second contained PHEA-fenoprofen conjugate equivalent to 43.40 µg/ml fenoprofen (0.155 g PHEA-fenoprofen conjugate). The maximum absorption of fenoprofen in both solutions was measured. The absorption of the fenoprofen solution was measured to be 0.65 at 272 nm (illustrated

in figure 7.13) and the absorption of fenoprofen in the PHEA-fenoprofen conjugate solution was measured to be 0.66 at 274 nm (illustrated in figure 7.14).

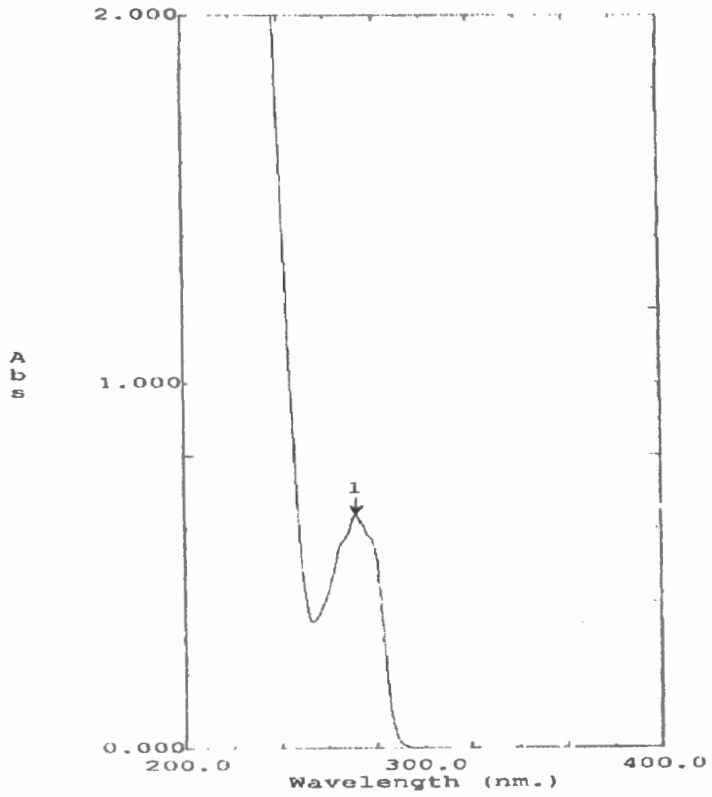


Figure 7.13: UV spectrum of fenoprofen (43.40 μg/ ml)

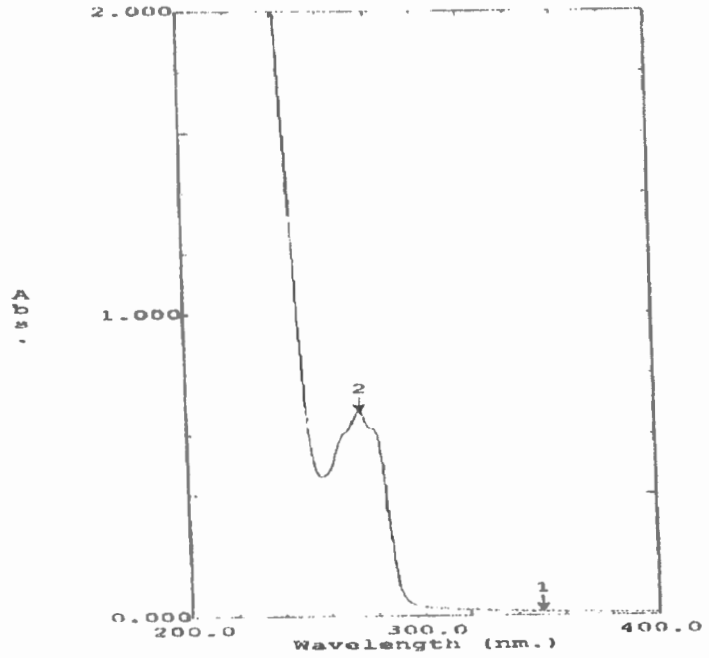


Figure 7.14: UV spectrum of PHEA-fenoprofen conjugate(concentration equivalent to 43.40 μg/ ml fenoprofen free drug)

7.5.2.2 Kinetic parameter calculations

The average concentration fenoprofen of released over a period of 72 hours are given in table 7.11.

Table 7.11: Average concentration fenoprofen released from PHEA within 72 hours at pH 10 (60 °C).

Time (hours)	Average concentration (µg/ml)
0	0
2	74.94
4	130.52
6	164.96
8	185.70
10	193.03
15	208.97
20	209.41
24	209.35
30	210.62
48	210.37
72	210.53

In order to calculate the chemical rate constant (k) for the reaction it is necessary to plot (figure 7.14) $\ln[C_{max}-C_t]$ versus time (data given in table 7.12) Maximum average concentration of fenoprofen released (C_{max}) within 72 hours can be obtained by calculating the average fenoprofen present during 30, 48 and 72 hours (C_{max} = 210.50 µg/ml).

Table 7.12: ln (C_{max}-C_t) and time (hours) data for the calculation of k.

Time (hours)	ln (C_{max}-C_t)
2	4.91
4	4.38
6	3.81
8	3.20
10	2.84

By using the data calculated in table 7.12, the plot of $\ln(C_{max}-C_t)$ versus time can be made (given in figure 7.15).

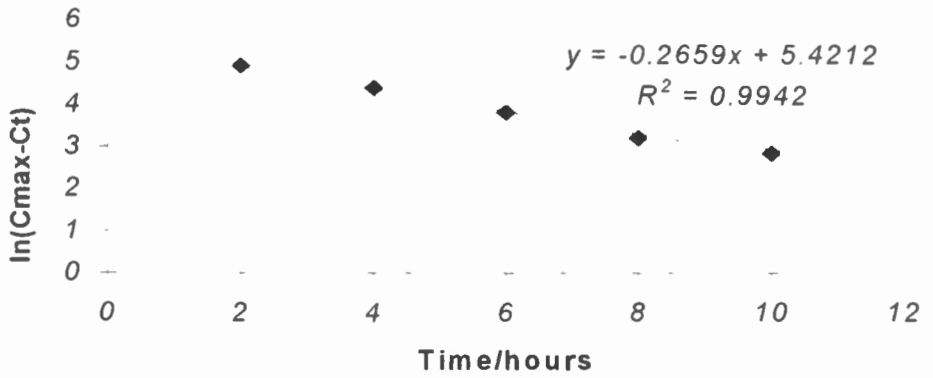


Figure 7.15: Plot of $\ln(C_{max}-C_t)$ versus time (hours).

Linear regression parameters for figure 7.14 are given in table 7.13.

Table 7.13: Linear regression parameters for plot $\ln(C_{max}-C_t)$ versus time (hours).

Regression Statistics					
Multiple R	0.997				
R Square	0.994				
Adjusted R Square	0.992				
Standard Error	0.074				
Observations	5				
	Coefficients	Standard Error	t Stat	P-value	Lower 95%
Intercept	5.421	0.078	69.478	6.5706E-06	5.172
X Variable 1	-0.265	0.011	-22.60	0.0001	-0.303

The chemical rate constant (k) is equal to the slope of plot $\ln(C_{max}-C_t)$ versus time and is equal to 0.2659 $hour^{-1}$.

The half life (paragraph 5.3.1) of this hydrolysis reaction can be calculated with equation 6.15:

$t_{½} = 0.693/0.2659$

$= 2.606$ hours.

7.5.3 Fenoprofen release from PHEA at 37°C at pH 10

The same standard curve for fenoprofen was also used (given in appendix 1). Fenoprofen release from PHEA is illustrated in figure 7.16 and values obtained during hydrolysis given in appendix 2. The fenoprofen calcium control sample showed no degradation through the experiment.

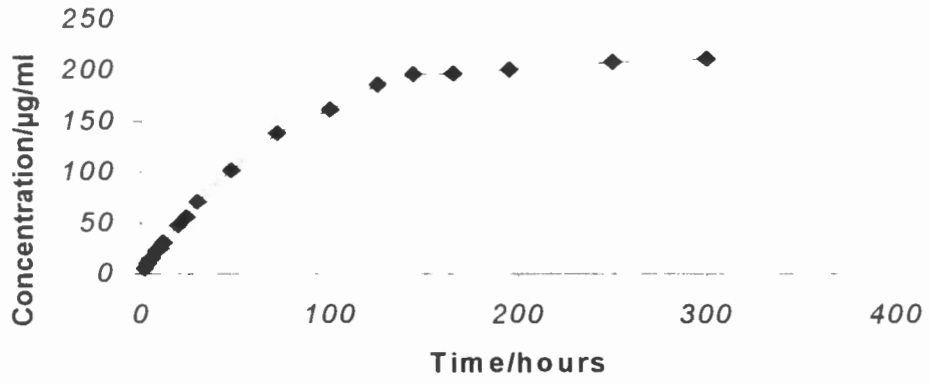


Figure 7.16: A graphical presentation of the concentration fenoprofen released against time at 37°C in boric acid buffer (pH 10)

7.5.3.1 Kinetic parameter calculations

The average concentration of fenoprofen released over a period of 300 hours is given in table 7.14.

Table 7.14: Average concentration of fenoprofen released from PHEA within 300 hours at pH 10 (37 °C).

Time (hours)	Average concentration (µg/ml)
2	4.23
4	9.94
6	15.02
8	21.61
10	24.08
12	29.99
20	47.33
24	55.46
30	70.60

Table 7.14 (continued): Average concentration fenoprofen released from PHEA within 300 hours at pH 10 (37 °C).

Time (hours)	Average concentration (µg/ml)
48	101.98
72	138.11
144	196.04
165	198.44
250	208.26
300	211.47

In order to calculate the chemical rate constant (k) for the reaction it is necessary to plot (figure 7.16) $\ln[C_{max}-C_t]$ versus time (data given in table 7.15) Maximum average concentration fenoprofen released (C_{max}) within 300 hours can be obtained by taking the amount fenoprofen released after 300 hours as C_{max}, (C_{max}=211.47 µg/ml).

Table 7.15: $\ln (C_{max}-C_t)$ and time (hours) data for the calculation of k.

Time (hours)	$\ln (C_{max}-C_t)$
2	5.33
4	5.31
6	5.28
8	5.24
10	5.23
12	5.20
20	5.10
24	5.04
30	4.94
48	4.69
72	4.29
144	2.73

By using the data calculated in table 7.15, the plot of $\ln(C_{max}-C_t)$ versus time can be made (given in figure 7.17).

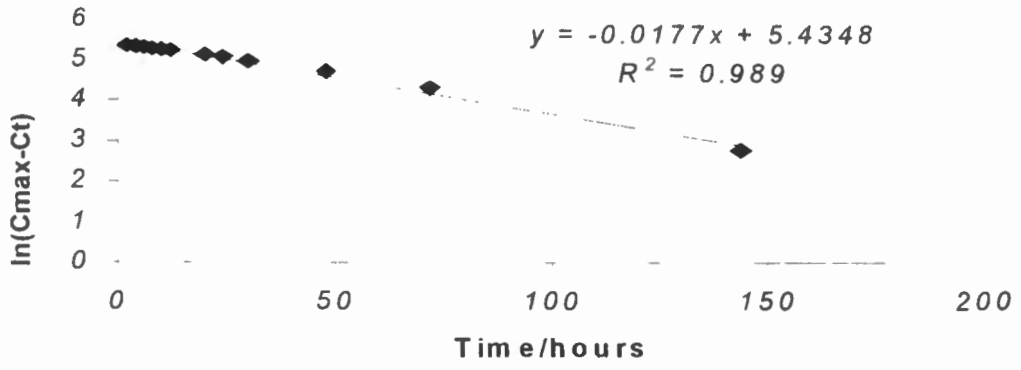


Figure 7.17: Plot of $ln(C_{max}-C_t)$ versus time (hours).

Linear regression parameters for figure 7.16 are given in table 7.16.

Table 7.16: Linear regression parameters for plot $ln(C_{max}-C_t)$ versus time (hours).

Regression Statistics						
Multiple R	0.994					
R Square	0.989					
Adjusted R Square	0.987					
Standard Error	0.079					
Observations	12					
	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper 95%
Intercept	5.434	0.029	183.351	5.72E-19	5.368	5.500
X Variable 1	-0.0176	0.0005	-30.051	3.9E-11	-0.018	-0.016

The chemical rate constant (k) is equal to the slope of plot $ln(C_{max}-C_t)$ versus time and is equal to 0.0177 $hour^{-1}$.

The half life (paragraph 5.3.1) of this hydrolysis reaction can be calculated with equation 6.15:

$t_{½} = 0.693/0.0177$

$= 39.15$ hours.

7.5.3.2 Activation energy calculation for fenoprofen release from PHEA (pH 10)

Activation energy for can be calculated by using the Arrhenius equation (equation 7.1):

$$\ln k_2 - \ln k_1 = (E_a / RT_1T_2)(T_2 - T_1) \quad (7.1)$$

where:

k_1 = 0.2659 $hour^{-1}$

k_2 = 0.0177 $hour^{-1}$

T_1 = 60 °C

T_2 = 37 °C

R = (universal gas constant = 8.31434 J. mol. K^{-1})

E_a = activation energy

With substitution of the above values into equation 7.1, activation energy was calculated to be 100.6 kJ. mol^{-1}.

7.5.4 Fenoprofen release from PHEA at 37 °C at pH 1.1

The standard curve used to calculate the obtained fenoprofen concentrations, the values obtained during hydrolysis and a representative chromatogram of fenoprofen released during hydrolysis are given in appendix 3. A graphical presentation of the concentration of fenoprofen released over time is given in figure 7.18. A fenoprofen sample was injected three times (after 24 hours, 100 hours and 800 hours) during the 800 hour period. The fenoprofen calcium control sample showed no degradation throughout the whole experiment.

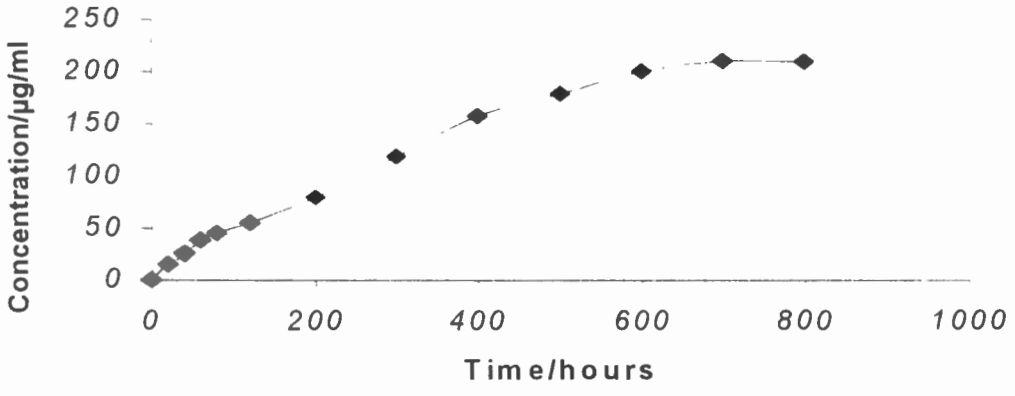


Figure 7.18: A graphical presentation of the concentration fenoprofen released against time at 37°C in glycine buffer (pH 1.1)

7.5.4.1 Kinetic parameter calculation

The average concentration of fenoprofen released over a period of 800 hours is given in table 7.17.

Table 7.17: Average concentration of fenoprofen released from PHEA within 800 hours at pH 1.1 (37 °C).

Time (hours)	Average concentration (µg/ml)
0	0
20	14.70
40	24.56
60	37.75
80	44.61
120	54.66
200	79.76
300	118.44
400	157.08
500	178.49
600	200.18
700	209.97
800	209.73

In order to calculate the chemical rate constant (k) for the reaction it is necessary to plot (figure 7.18) $\ln[C_{max}-C_t]$ versus time (data given in table 7.17) Maximum average concentration fenoprofen released (C_{max}) within 800 hours can be obtained by taking the amount fenoprofen released after 600 hours as C_{max}, (C_{max}=209.73 µg/ml).

Table 7.18: ln ($C_{max}-C_t$) and time (hours) data for the calculation of k.

Time (hours)	ln ($C_{max}-C_t$)
20	5.27
40	5.22
60	5.14
80	5.11
120	5.04
200	4.87
300	4.51
400	3.96
500	3.44
600	2.25

By using the data calculated in table 7.13, the plot of $\ln(C_{max}-C_t)$ versus time can be made (given in figure 7.19).

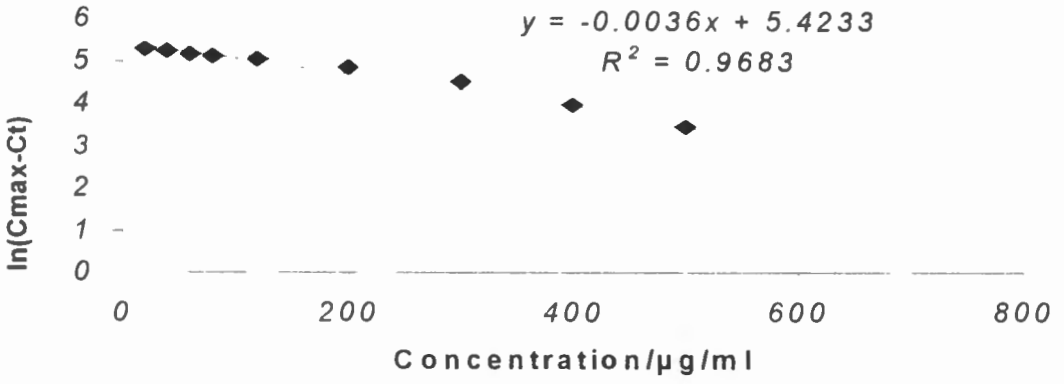


Figure 7.19: Plot of $\ln(C_{max}-C_t)$ versus time (hours).

Linear regression parameters for figure 7.18 are given in table 7.19.

Table 7.19: Linear regression parameters for plot $\ln(C_{max}-C_t)$ versus time (hours).

Regression Statistics						
Multiple R	0.98					
R Square	0.96					
Adjusted R Square	0.96					
Standard Error	0.12					
Observations	9					
	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper 95%
Intercept	5.423	0.0624	86.797	7.1E-12	5.275	5.57102
X Variable 1	-0.003	0.0002	-14.62	1.67E-06	-0.004	-0.003

The chemical rate constant (k) is equal to the slope of plot $\ln(C_{max}-C_t)$ versus time and is equal to 0.0036 $hour^{-1}$.

The half life (paragraph 5.3.1) of this hydrolysis reaction can be calculated with equation 6.15:

$t_{½}$= 0.693/0.0036

= 192.5 hours.

7.5 PHEA-fenoprofen conjugate powder dissolutions

7.5.1 Powder dissolution in simulated gastric juice

No fenoprofen was released in a period of 12 hours. A representative chromatogram of fenoprofen released during dissolution is given in appendix 4.

7.5.2 Powder dissolution in simulated intestinal fluid

No fenoprofen was released in a period of 12 hours. A representative chromatogram of fenoprofen released during dissolution is given in appendix 5.

Chapter 8: Conclusions

This study has shown that:

- PHEA-fenoprofen conjugate shows different physical properties than fenoprofen calcium. This was shown by the fact that PHEA-fenoprofen conjugate shows a different melting point, different x-ray powder diffractometric pattern, different solubility profiles and a different infrared spectrum than fenoprofen calcium. Fenoprofen is in other words really chemically bound to PHEA and not merely dispersed or incorporated in PHEA. This can also be confirmed by the calculation of the activation energy (100.6 $kJ.mol^{-1}$) for fenoprofen release from PHEA. This value is typically the value needed if a sigma bond wants to be broken.

- Analytical methods used for fenoprofen calcium can be adapted to analyse the PHEA-fenoprofen conjugate. HPLC method performance parameters for fenoprofen can be improved by substituting a C_{18} column with a C_8 column.

- Poor solubility of the conjugate in popular solvents used in analytical chemistry makes it difficult to analyse PHEA-fenoprofen conjugate. HPLC methods proved to be the only method that can be used to determine the amount free fenoprofen (fenoprofen released from PHEA). This HPLC method can only determine the amount fenoprofen in the conjugate after hydrolysis (simply injecting PHEA-fenoprofen conjugate before hydrolysis, shows no chromatogram peak).

- Spectrophotometry can only determine the total amount of fenoprofen present in the conjugate (free fenoprofen and fenoprofen bound to PHEA). Spectrophotometric methods can thus only be used in fenoprofen drug loading determinations and can not be used in hydrolysis or dissolution studies.

- The ratio fenoprofen: PHEA was calculated to be 1: 4.7. This ratio is too high if oral dosages are to be given. This will mean that 1.4 g PHEA-fenoprofen conjugate will have to be administered in order to obtain a single fenoprofen dose of 300 mg.

- Fenoprofen release from PHEA follows second-order kinetics, but because the amount OH^- (or H^- in the case of acidic hydrolysis), is not present in limited amounts, the reaction can be classified as a pseudo first-order reaction. The chemical rate constants (k) for fenoprofen release in boric acid buffer was calculated to be 0.26 $hours^{-1}$ in 60 °C and 0.02

hours^{-1} in 37 °C. These values are indicative of delayed release of fenoprofen from PHEA, which will most likely prolong fenoprofen's pharmacological action *in vivo*. This will also have to be investigated further in future.

- The chemical rate constant (k) for fenoprofen release in glycine buffer solution was calculated to be 0.0036 hour^{-1}. This value is indicative of delayed release of fenoprofen in acidic medium, which will make fenoprofen release in gastric acid very difficult if not impossible. .

- The phenomenon that no fenoprofen was released from PHEA in simulated gastric or intestinal fluid will make oral dosages difficult if not impossible. Intravenous dosage forms will have to be considered and will make exiting futuristic studies.

Appendix

Appendix 1:

Table 1: Standard curve values for fenoprofen used to calculate drug loading

Concentration fenoprofen (μg/ml)	Area under curve (mV-sec)
260.4	2456257
130.2	1222029
65.1	60944
52.1	486787
43.4	408277
34.7	323028
28.9	272512

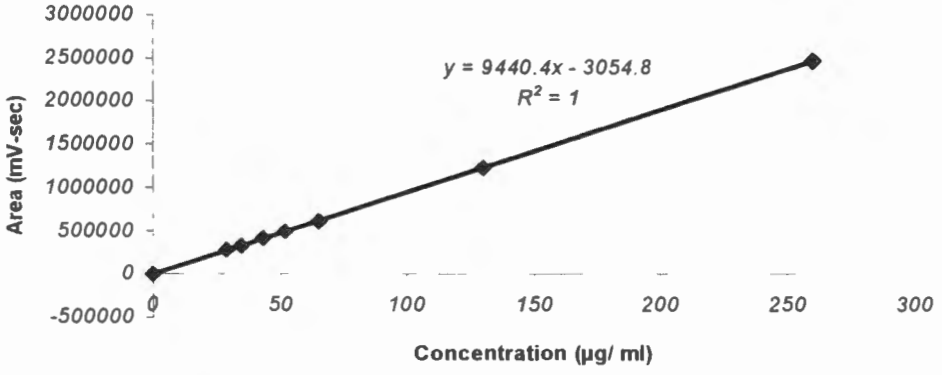


Figure 1: Graphical presentation of linearity plot of fenoprofen used in 60 °C hydrolysis (pH 10)

Table 2: Concentration of fenoprofen released from fenoprofen-PHEA drug ester during different time-intervals.

Number of experimental repeats	Time (hours)	Average area under curve (mV-sec)	Average concentration of 5 injections	% RSD
1	2	701163	74.60	0.28
2	2	702906	74.78	0.23
3	2	711829	75.73	0.33
4	2	7133047	75.86	0.33
5	2	698060	74.27	0.24
6	2	699532	74.42	0.31
1	4	1233192	130.95	0.22
2	4	1219528	129.51	0.43
3	4	1209073	128.40	0.37
4	4	1215626	129.09	0.51
5	4	1281198	136.06	0.55
6	4	1216241	129.16	0.19
1	6	1528533	162.24	0.15
2	6	1495738	165.70	0.15
3	6	1561183	168.43	0.42
4	6	1587030	170.27	0.26
5	6	1604375	164.33	0.18
6	6	1548298	158.76	0.37
1	8	1725683	183.12	0.85

Table 2 (continued): Concentration of fenoprofen released from fenoprofen-PHEA drug ester during different time-intervals.

Number of experimental repeats	Time (hours)	Average area under curve (mV-sec)	Average concentration of 5 injections	% RSD
2	8	1760347	186.79	1.10
3	8	1749607	185.66	0.16
4	8	1720409	182.56	0.25
5	8	1751713	185.88	0.27
6	8	1792589	190.21	0.32
1	10	1855929	196.92	0.5
2	10	1826088	193.76	0.38
3	10	1789727	189.91	0.18
4	10	1824639	193.60	0.70
5	10	1790273	189.96	0.40
6	10	1828731	194.04	0.24
1	15	1960505	208.00	0.48
2	15	1974799	209.51	0.37
3	15	1974799	209.51	0.37
4	15	1964417	208.41	0.50
5	15	1962222	208.18	0.20
6	15	1981336	210.20	0.37
1	20	1981336	210.20	0.37
2	20	1964854	208.46	0.16
3	20	1975799	209.62	0.35
4	20	1962815	208.24	0.41
5	20	1978744	209.93	0.25
6	20	1979520	210.01	0.18

Table 2 (continued): Concentration of fenoprofen released from fenoprofen-PHEA drug ester during different time-intervals.

Number of experimental repeats	Time (hours)	Average area under curve (mV-sec)	Average concentration of 5 injections	% RSD
1	24	1962423	208.20	0.35
2	24	1991338	211.26	0.25
3	24	1986704	210.77	0.14
4	24	1963321	208.29	0.61
5	24	1961872	208.14	0.43
6	24	1974589	209.49	0.26
1	30	1977108	209.75	0.45
2	30	1992737	211.41	0.60
3	30	1987673	210.87	0.25
4	30	1984797	210.57	0.28
5	30	1979188	209.97	0.61
6	30	1990695	211.19	0.32
1	48	1993061	211.44	0.37
2	48	1988695	210.98	0.44
3	48	1992545	211.39	0.29
4	48	1971799	209.19	0.29
5	48	1971248	209.13	0.12
6	48	1980473	210.11	0.40

Table 2 (continued): Concentration of fenoprofen released from fenoprofen-PHEA drug ester during different time-intervals.

Number of experimental repeats	Time (hours)	Average area under curve (mV-sec)	Average concentration of 5 injections	% RSD
1	72	2017133	213.99	0.40
2	72	1988923	211.01	0.19
3	72	1964014	208.37	0.29
4	72	2003647	212.57	0.59
5	72	1964629	208.43	0.47
6	72	1968358	208.83	0.43

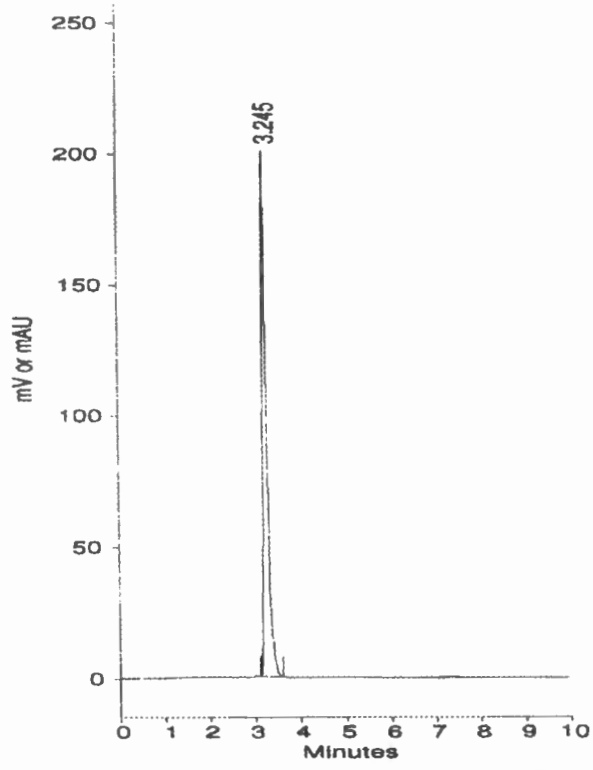


Figure 2: A representative chromatogram of fenoprofen released during hydrolysis (60°C for 72 hours)

Appendix 2:

Table 1: Concentration of fenoprofen released from fenoprofen-PHEA drug ester during different time-intervals at 37 °C (pH 10).

Number of experimental repeats	Time (hours)	Average area under curve (mV-sec)	Average concentration of 5 injections	% RSD
1	2	43113	4.89	0.23
2	2	48684	5.48	0.69
3	2	46251	5.22	0.31
4	2	41022	4.67	0.24
5	2	44204	5.01	0.62
6	2	45225	5.11	0.23
1	4	88817	9.73	0.19
2	4	88993	9.75	0.33
3	4	89596	9.81	0.19
4	4	88612	9.71	0.55
5	4	93620	10.24	0.60
6	4	95233	10.41	0.47
1	6	136968	14.83	0.27
2	6	149953	16.21	0.17
3	6	136425	14.77	0.41
4	6	136521	14.78	0.16
5	6	136025	14.73	0.09
6	6	136431	14.78	0.08
1	8	203965	21.93	0.11
2	8	203881	21.92	0.13
3	8	195530	21.04	0.44
4	8	204779	22.02	0.38

Table 1 (continued): Concentration of fenoprofen released from fenoprofen-PHEA drug ester during different time-intervals at 37 °C (pH 10).

Number of experimental repeats	Time (hours)	Average area under curve (mV-sec)	Average concentration of 5 injections	% RSD
5	8	198247	21.32	0.08
6	8	199117	21.42	0.27
1	10	223749	24.02	0.41
2	10	223801	24.03	0.13
3	10	234058	25.12	0.18
4	10	224139	24.07	0.16
5	10	225738	24.24	0.12
6	10	213896	22.98	0.35
1	12	295400	31.61	0.03
2	12	289003	30.94	0.20
3	12	285933	30.61	0.28
4	12	275386	29.49	0.21
5	12	274634	29.41	0.20
6	12	288651	30.90	0.04
1	20	449141	47.90	0.25
2	20	444973	47.46	0.08
3	20	442262	47.17	0.24
4	20	444555	47.41	0.18
5	20	437716	46.69	0.38
6	20	443740	47.33	0.36
1	24	520227	55.43	0.54
2	24	528620	56.32	0.54
3	24	500188	53.31	0.21
4	24	528605	56.32	0.34

Table 1 (continued): Concentration of fenoprofen released from fenoprofen-PHEA drug ester during different time-intervals at 37 °C (pH 10).

Number of experimental repeats	Time (hours)	Average area under curve (mV-sec)	Average concentration of 5 injections	% RSD
5	24	527334	56.18	0.28
6	24	518164	55.21	0.26
1	30	646926	68.85	0.57
2	30	676007	71.93	0.08
3	30	666825	70.96	0.32
4	30	649340	69.11	0.32
5	30	677980	72.14	0.39
6	30	688901	73.30	0.06
1	48	943392	100.25	0.24
2	48	941810	100.09	0.07
3	48	971781	103.26	0.43
4	48	968656	102.93	0.38
5	48	964853	102.53	0.44
6	48	967758	102.84	0.27
1	72	1314465	139.56	0.67
2	72	1308206	138.90	0.21
3	72	1284256	136.36	0.36
4	72	1311696	139.27	0.28
5	72	1285350	136.48	0.37
6	72	1305897	138.65	0.26
1	100	1535589	162.99	0.06
2	100	1514425	160.74	0.49
3	100	1513884	160.69	0.38

Table 1 (continued): Concentration of fenoprofen released from fenoprofen-PHEA drug ester during different time-intervals at 37°C (pH 10).

Number of experimental repeats	Time (hours)	Average area under curve (mV-sec)	Average concentration of 5 injections	% RSD
4	100	1528896	162.28	0.18
5	100	1504499	159.69	0.04
6	100	1539846	163.44	0.03
1	125	1748621	185.55	0.15
2	125	1755919	186.32	0.59
3	125	1747758	185.46	0.42
4	125	1744762	185.14	0.12
5	125	1742465	184.90	0.11
6	125	1756558	186.39	0.12
1	144	1847092	195.98	0.08
2	144	1844394	195.70	0.28
3	144	1844196	195.68	0.61
4	144	1855516	196.87	0.03
5	144	1848637	196.15	0.43
6	144	1846115	195.88	0.23
1	165	1850442	196.34	0.17
2	165	1863468	197.72	0.11
3	165	1850804	196.38	0.17
4	165	1850338	196.33	0.01
5	165	1856410	196.97	0.22
6	165	1850445	196.34	0.01
1	195	1895226	201.08	0.20
2	195	1896805	201.25	0.16
3	195	1892763	200.82	0.31

Table 1 (continued): Concentration of fenoprofen released from fenoprofen-PHEA drug ester during different time-intervals at 37°C (pH 10).

Number of experimental repeats	Time (hours)	Average area under curve (mV-sec)	Average concentration of 5 injections	% RSD
4	195	1880210	199.49	0.12
5	195	1888953	200.49	0.026
6	195	1884396	199.93	0.32
1	250	1966751	208.66	0.77
2	250	1964557	208.42	0.10
3	250	1959984	207.94	0.08
4	250	1963447	208.31	0.06
5	250	1968145	208.80	0.06
6	250	1954887	207.40	0.05
1	300	1986607	210.76	0.06
2	300	2004452	212.65	0.63
3	300	1995126	211.66	0.13
4	300	1991472	211.28	0.63
5	300	1990078	211.13	0.13
6	300	1992225	211.36	0.06

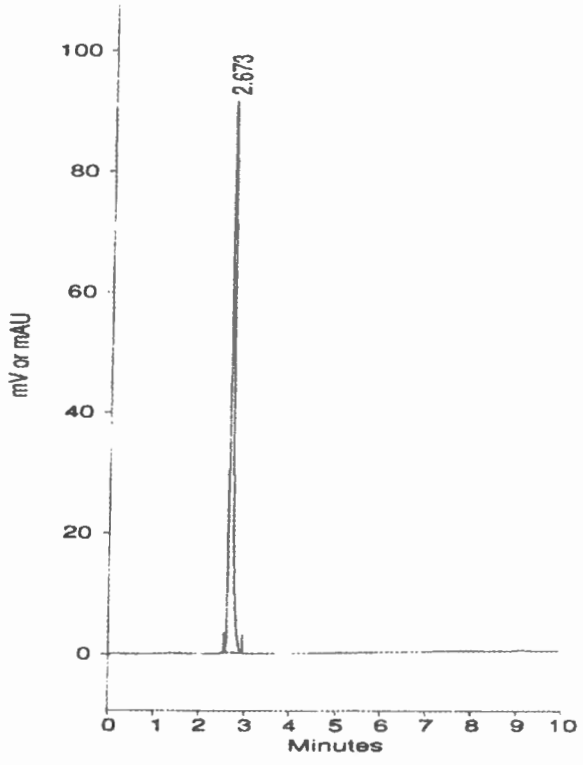


Figure 1: A representative chromatogram of fenoprofen release at 37 °C and pH 10 for 300 hours

Appendix 3:

Table 1: Standard curve values for fenoprofen used to calculate fenoprofen release at pH 1.1 (37 °C).

Concentration fenoprofen (µg/ml)	Area under curve (mV-sec)
260.4	2008528
130.2	989184
65.1	425129
52.1	315402
43.4	257750
34.7	206023
28.9	119307

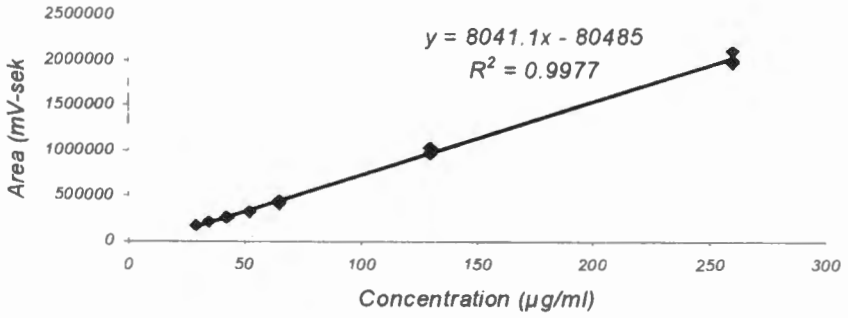


Figure 1: Graphical presentation of linearity plot of fenoprofen used for hydrolysis at 37 °C.

Table 2: Concentration of fenoprofen released from fenoprofen-PHEA drug ester during different time-intervals at 37 °C (pH 1.1).

Number of experimental repeats	Time (hours)	Average area under curve (mV-sec)	Average concentration of 5 injections	% RSD
1	20	40222	15.011	0.92
2	20	39973	14.980	0.46
3	20	34197	14.262	0.12
4	20	33797	14.212	0.82
5	20	39010	14.861	0.60
6	20	38995	14.859	0.32
1	40	119741	24.900	0.11
2	40	119887	24.919	0.54
3	40	118557	24.753	0.98
4	40	117918	24.674	0.78
5	40	120587	25.006	0.85
6	40	105402	23.117	0.95
1	60	225379	38.038	0.65
2	60	231846	38.842	0.54
3	60	209949	36.119	0.21
4	60	230041	38.617	0.28
5	60	220796	37.468	0.95
6	60	220500	37.431	0.54
1	80	275321	44.248	0.49
2	80	281370	45.001	0.33
3	80	272576	43.907	0.74
4	80	275612	44.285	0.66
5	80	280331	44.872	0.19

Table 2 (continued): Concentration fenoprofen released from fenoprofen-PHEA drug ester during different time-intervals at 37 °C (pH 1.1).

Number of experimental repeats	Time (hours)	Average area under curve (mV-sec)	Average concentration after 5 injections	% RSD
6	80	284072	45.337	0.17
1	120	356006	54.28	0.24
2	120	369062	55.90	0.38
3	120	354182	54.06	0.69
4	120	357538	54.47	0.47
5	120	359592	54.72	0.58
6	120	357623	54.48	0.14
1	200	548645	78.23	0.25
2	200	551338	78.57	0.69
3	200	565724	80.36	0.47
4	200	592449	83.68	0.58
5	200	559412	79.57	1.19
6	200	563520	80.08	0.28
1	300	882645	119.77	0.17
2	300	849802	115.69	0.39
3	300	883153	119.83	0.75
4	300	881315	119.61	0.95
5	300	882072	119.70	0.39
6	300	852843	116.07	0.27
1	400	1176917	156.37	0.29
2	400	1179488	156.69	0.57
3	400	1178849	156.61	0.05
4	400	1182078	157.01	0.58

Table 2 (continued): Concentration of fenoprofen released from fenoprofen-PHEA drug ester during different time-intervals at 37°C (pH 1.1).

Number of experimental repeats	Time (hours)	Average area under curve (mV-sec)	Average concentration of 5 injections	% RSD
5	400	1185518	157.44	0.55
6	400	1192968	158.36	0.67
1	500	1324666	174.74	0.83
2	500	1364353	179.68	0.52
3	500	1367643	180.09	0.36
4	500	1359686	179.10	0.32
5	500	1356058	178.65	0.68
6	500	1356264	178.67	048
1	600	1502430	196.85	1.20
2	600	1535302	200.94	1.98
3	600	1507835	197.52	0.45
4	600	1549585	202.71	0.68
5	600	1530696	200.36	0.87
6	600	1549632	202.72	0.63
1	700	1504141	197.06	0.54
2	700	1603348	209.40	0.52
3	700	1602197	209.26	0.98
4	700	1606121	209.74	0.78
5	700	1607992	209.42	0.99
6	700	1610191	209.98	0.83

Table 2 (continued): Concentration of fenoprofen released from fenoprofen-PHEA drug ester during different time-intervals at 37°C (pH 1.1).

Number of experimental repeats	Time (hours)	Average area under curve (mV-sec)	Average concentration of 5 injections	% RSD
1	800	1624775	212.07	0.35
2	800	1609341	210.15	0.66
3	800	1575402	205.93	1.22
4	800	1607447	209.91	0.87
5	800	1614012	210.73	0.59
6	800	1605125	209.62	0.19

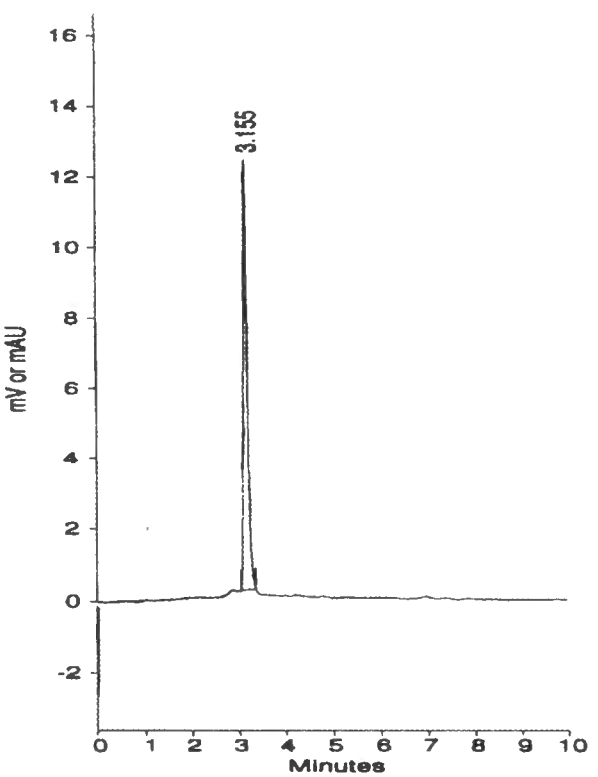


Figure 1: A representative chromatogram of fenoprofen release at 37 °C and pH 1.1

Appendix 4:

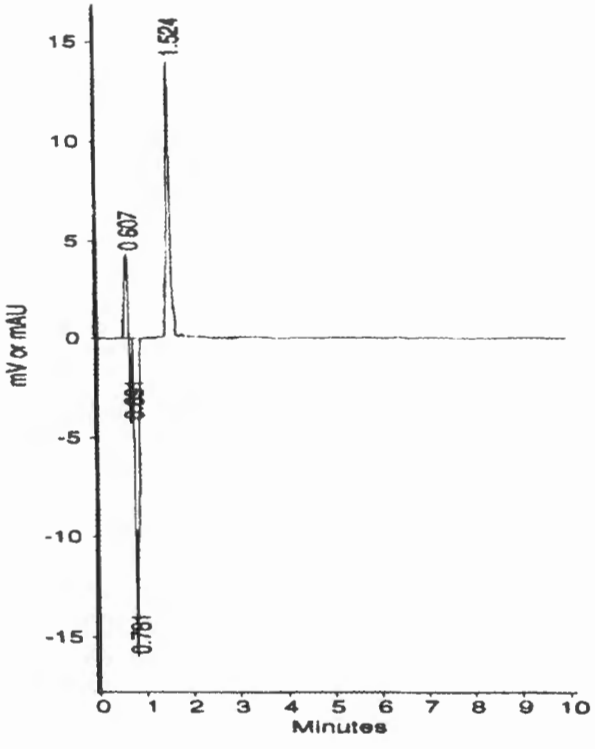


Figure 1: A representative chromatogram of fenoprofen release in simulated gastric juice (pH 1.2)

Appendix 5:

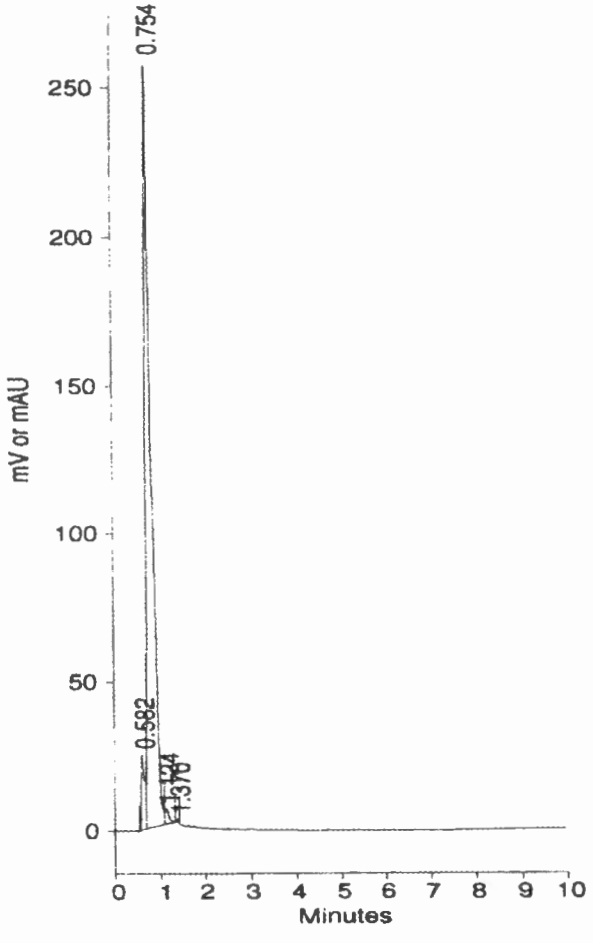


Figure 1: A representative chromatogram of fenoprofen release in simulated intestinal fluid (pH 6.8)

Summary

The term "prodrug" refers to a pharmacologically inactive compound that is converted to an active drug by a metabolic biotransformation. A nonenzymatic process, such as hydrolysis, can activate a prodrug *via* several routes.

Prodrugs are mostly used to improve a drug's physical and chemical properties in order to optimise drug delivery. Not only can prodrugs prolong therapeutic effect, but side-effects can also be minimised. Macromolecular prodrugs are one type of prodrug that have drawn a considerable amount of attention the last few years. Macromolecular prodrugs differ from ordinary carrier-linked prodrugs in the sense that the drug carrier is a macromolecule. Of all the macromolecular drug carriers, poly[α,β-(*N*-2-hydroxyethyl-DL-aspartamide)] (PHEA) is the most promising drug carrier because it is hydrophilic, non-toxic, non-antigenic, biodegradable in the presence of several enzymes, and produced easily and at low cost. These types of carriers can be used as possible drug carriers as well as plasma expanders, in order to decrease the required dose, dosage intervals and drug toxicity.

Long term usage (with frequent high dosages) of the NSAIDs can lead to severe blood-disorders and the formation of peptic ulcers. These two problems encountered with NSAID therapy cause patients to quit NSAID treatment, and rather live with the illness than living with the side-effects.

Macromolecular PHEA prodrugs is one attempt that can be made to improve major disadvantages of NSAID treatment, and because the advantages of the macromolecular prodrugs speak for themselves, it is worth trying to formulate some of the NSAIDs most commonly used over long-term into macromolecular prodrugs. If these drug carriers can be used to prolong pharmacological action and lower drug toxicity, some of the older NSAIDs (with limited usage at present) can again be used.

Before any new synthesised prodrug can be used, it is necessary to develop new methods of analysis. These methods must be fully validated in order to quantitate the prodrug in different matrixes, depending on the purpose of the analysis. It is therefor essential to utilise methods that can selectively, accurately and precisely quantitate the prodrug in various matrixes with an acceptable limit of detection and limit of quantitation.

The aim of this study was therefore to develop an HPLC and a spectrophotometric method for the analysis of a PHEA-fenoprofen conjugate and to compare these two methods with each other. To fully validate the developed HPLC method, to determine fenoprofen drug loading in the conjugate by means of hydrolysis, to determine the release of fenoprofen from the conjugate under certain simulated biological conditions. To determine and describe the kinetics of fenoprofen release from the conjugate, and to determine the relevant physical properties influencing the analysis of the conjugate.

This study has shown that PHEA-fenoprofen conjugate has different physical properties than fenoprofen calcium, and proves that fenoprofen is really chemically bound to PHEA and not merely dispersed or incorporated in PHEA. Analytical methods used for fenoprofen can be adapted for PHEA-fenoprofen conjugate analysis. Poor solubility of the conjugate in popular solvents used in analytical chemistry makes it difficult to analyse PHEA-fenoprofen conjugate. Fenoprofen release from PHEA follows pseudo first-order kinetics. Calculated reactions constants (k) are indicative of delayed release of fenoprofen from PHEA, which will most likely prolong fenoprofen's pharmacological action *in vivo*, and can be investigated further in future.

Opsomming

Die term "progeneesmiddel" verwys na 'n farmakologies onaktiewe verbinding wat deur metaboliese transformasie omgeskakel word na die aktiewe geneesmiddel. 'n Nie-ensimatiese proses, byvoorbeeld hidrolise, kan 'n progeneesmiddel deur middel van verskeie roetes aktiveer.

Progeneesmiddels word meestal gebruik om 'n geneesmiddel se fisiese en chemiese eienskappe te verbeter en sodoende die werking van die geneesmiddel so effektief as moontlik te maak. Nie net kan 'n progeneesmiddel gebruik word om 'n geneesmiddel se terapeutiese effek te verbeter nie, maar ook om sekere newe-effekte uit te skakel of te verminder.

Makromolekulêre progeneesmiddels verskil van gewone progeneesmiddels in die sin dat die draer molekule 'n makromolekule is. Van al die makromolekulêre draers beskikbaar, is poly[α,β-(*N*-2-hidroksi-etiel-DL-aspartamied)] (PHEA) die mees belowend. PHEA se voordele sluit in dat hierdie verbinding uiters hidrofilies is, nie-toksies, *in vivo* maklik afbreekbaar is en maklik teen lae koste gesintetiseer kan word.

Langtermyngebruik (gereelde hoë dosisse) van die nie steroïed anti-inflammatoriese middels (NSAIMs) kan lei tot ernstige bloedafwykings en die vorming van potensieel lewensbedreigende peptiese ulkusse. Hierdie twee probleme met die NSAIMs lei daartoe dat pasiënte hul behandelings met NSAIMs staak en eerder lewe met die siekte as met die newe-effekte.

Makromolekulêre PHEA progeneesmiddels is een poging wat gebruik kan word om sekere van die NSAIMs se newe-effekte te help verbeter. As hierdie draers gebruik kan word om van die ergste newe-effekte van die NSAIMs te verbeter kan baie van dié middels (met beperkte gebruik tans) weer terapeuties aan gewend.

Voordat enige nuwe gesintetiseerde progeneesmiddel gebruik kan word, is dit eers nodig om nuwe metodes van analise te ontwikkel. Hierdie metodes moet in staat wees om die progeneesmiddel te kan kwantifiseer in verskillende eksperimente met verskillende doelwitte. Hierdie metodes moet die geneesmiddel selektief, akkuraat en met 'n goeie limiet van kwantifikasie kan analiseer.

In hierdie studie is:

- 'n HDVC en spektrofotometriese metode ontwikkel en hierdie metodes met mekaar te vergelyk.
- Die HDVC metode volledig gevalideer, om sodoende hierdie metode te kan gebruik om die hoeveelheid fenoprofen in die konjugaat te bepaal deur middel van hidrolise en
- om die kinetika van fenoprofen vrystelling vanuit PHEA te bepaal.

Hierdie studie het getoon dat die PHEA-fenoprofen konjugaat se fisiese eienskappe verskil van dié van fenoprofenkalsium. Swak oplosbaarheid van die konjugaat bemoeilik analise omdat algemene oplosmiddels nie gebruik kan word nie. Fenoprofen se vrystelling van PHEA volg pseudo-eerste orde kinetika. Berekende reaksiekonstantes (k) toon 'n vertraagde vrystelling van fenoprofen aan, wat waarskynlik fenoprofen se terapeutiese werking *in vivo* gaan beïnvloed. Hierdie verskynsel kan in die toekoms verder ondersoek word.

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IF PEOPLE KNEW HOW HARD I WORKED TO GAIN MY MASTERY, IT WOULDN'T SEEM SO WONDERFUL AT ALL.

Michelangelo